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Obituary Notice

ERNST FREUND, 1863–1946

Professor Ernst Freund died in London on 2 June 1946 at the advanced age of 83. Born in Vienna, he studied medicine there, obtaining his M.D. degree when he was only 22. In the middle of the nineties of the last century he was appointed Director of the Biochemical Department of the 'Rudolf-Spital', which hospital he served until he reached the retiring age, for part of the time as Professor of Pathological Chemistry in the University of Vienna.

This period of Freund's long life coincided with a great epoch in European and Viennese medical science to which he made many valuable contributions, such as the introduction of sodium citrate as an anticoagulant in blood transfusion (1891), a proposal which arose directly out of his accomplished study of the coagulation of the blood. Many of his discoveries and innovations so readily found acceptance that their authorship has not infrequently been lost sight of. It was his endeavour always to apply chemical technique to the study of pathological problems.

Freund's research work covered a wide field, but among his many interests may be singled out the physiology and pathology of digestion and the mechanism of absorption of nutrients through the intestinal wall, the importance to the organism of a normally balanced bacterial flora in the gut and methods of urine analysis. He devoted much time to the study of tuberculosis, but it was undoubtedly the problem of malignant growth which exercised for him the greatest fascination.

On this subject he was led in those early days to conclusions, many of which stimulated further work in this very difficult field. With his collaborator G. Kaminer he described the cytolytic reaction which he modified and revised in many respects in later years. The technique of this test was much too complicated to become a routine investigation. It is, however, fair to say that these highly original ideas have stimulated widespread search for a diagnostic reaction for cancer, a search which is still proceeding.

When Freund was approaching the retiring age, the Pearson Cancer Research Foundation was created by Mr F. F. A. Pearson to enable him to continue his work upon malignant disease, which he did until his last day, first at the Rudolf-Spital in Vienna and later in London, when the whole Foundation was transferred to England as a result of the Nazi invasion of Austria.

Freund's long lifetime has witnessed very great changes in biochemical outlook, theory and technical achievement. He belonged to an older generation of biochemists, a pioneer generation, if one may use the term, but his devotion to his subject and intellectual enthusiasm remained with him to the last. He has left to posterity a scientific inheritance of more than 200 papers. C. R.

The Respiration of *Mycobacterium phlei*

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(*Received* 27 *August* 1946)

Stephenson & Whetham (1922, 1924) found that *Mycobacterium phlei* growing in a synthetic medium utilized either glucose or lactate as the sole source of carbon. No breakdown product other than CO_2 was detected. Merrill (1930, 1931 *a*, *b*) likewise found no evidence of partial degradation in cultures. Ebina & Nakamura (1937) studied the oxidation of glucose by washed suspensions, noting the absence of aerobic glucolysis. The measurements of Edson & Hunter (1943), which show that both glucose and lactate are oxidized vigorously by washed suspensions of *Myco. phlei*, suggest that further investigation of the behaviour of these substrates might be profitable.

METHODS

Organisms and suspensions. Three strains of *Myco. phlei* were employed, no. 525 N.C.T.C. and two others apparently identical with it. The stock cultures were maintained on glycerinated Dorset's egg medium and transferred to meat infusion broth containing 5% of glycerol for several generations before use. On the 7th–9th day of incubation at 38° the bacteria were washed and suspended in distilled water by methods described previously (Edson & Hunter, 1943).

Non-viable preparations. (*a*) *Bacterial extracts* were prepared with the wet crushing mill of Booth & Green (1938). Bacteria (30 g.) were suspended as a creamy emulsion in 20 ml. of distilled water and crushed for 2·5 hr. About 40% of the cells were crushed, the fragments losing their acid-fast properties. The heavier debris was removed by centrifuging for 1 hr. at 4000 r.p.m. The supernatant liquid was yellowish brown and slightly opalescent.

(*b*) *Acetone powders.* Dry powders were made from washed bacteria by stirring and centrifuging three times with 10 vol. of cold acetone. The acetone powder was dried *in vacuo* and stored at 4°. Activity was retained for at least 5 weeks. Although acetone removes considerable amounts of pigment and lipids, the powder is yellow and does not wet readily. For manometric experiments it was converted into a uniform suspension either in distilled water or in buffer by shaking in a miniature stainless steel ball-mill (see Edson & Hunter, 1943). Suspensions usually contained 10–20 mg. of dry powder/ml.

Manometric measurements. Oxygen consumption, CO_2 production (aerobic and anaerobic) and bicarbonate were determined by the standard methods of Warburg. When HCN was used as inhibitor, the CO_2 absorbent was an appropriate mixture of KOH and KCN (Krebs, 1935). Substrates were added after equilibration. Acid substrates were used as neutral sodium salts.

Analytical methods

Determination of glucose. After a manometric experiment each cup was cooled in ice and the contents transferred to a graduated test-tube by means of a Pasteur pipette. The cup and side-bulb were washed twice with 1 ml. of distilled water, the washings being added to the test-tube in the same manner. The deproteinizing reagents (0·4 ml. 10% (w/v) sodium tungstate and 0·4 ml. 0·66 N-H_2SO_4), which also flocculated the bacteria, were added together with sufficient distilled water to make a total volume of 8·0 ml. The mixture was filtered through a Whatman no. 30 paper (4·25 cm.) and a 5 ml. sample used for analysis by the method of Shaffer & Somogyi (1933).

Determination of lactic acid. The manometric cups were cooled in ice and the contents transferred (by Pasteur pipette) to a 100 ml. volumetric flask. The washings, five lots each of 1 ml., were added in the same way, followed by 4 ml. 20% (w/v) $CuSO_4.5H_2O$ solution and 4 ml. $Ca(OH)_2$ suspension in succession and the volume brought to 100 ml. with distilled water. The copper-lime reagent removed the bacteria effectively. The mixture was filtered through a Whatman no. 1 paper (11 cm.) and the filtrate acidified by addition of 0·2 ml. of phosphoric acid (sp.gr. 1·7). An amount of 80 ml. was used for the determination of lactic acid by the method of Friedemann & Graeser (1933).

Detection of acetic acid. At the end of an experiment the bacterial suspension was treated with $\frac{1}{10}$ vol. of freshly prepared 7% (w/v) metaphosphoric acid and filtered through a Whatman no. 30 paper (4·25 cm.). Bacteria and dissolved proteins were thus removed. The acid filtrate was subjected to steam distillation in a micro-apparatus in which the volume (5 ml.) of the boiling liquid was kept almost constant. A volume of 20 ml. of distillate was collected, made faintly alkaline with a few drops of N-NH_4OH, and evaporated gently to 2 ml. on a hot water-bath. The concentrate was tested by the lanthanum-iodine reaction, 0·3 ml. of 5% lanthanum nitrate solution, 0·3 ml. of 0·01 N-ethanolic iodine solution and 0·2 ml. of N-NH_4OH being added in succession.

RESULTS

Oxygen consumption in the presence of carbohydrates, fatty acids and related substances

The spontaneous oxygen consumption of washed suspensions of *Myco. phlei* is fairly constant for different cultures; and the oxygen uptake is augmented by several substances, notably glucose and lactate, both of which cause a five-fold increase (Table 1). Mannose, trehalose, pyruvate and *l*(−)-malate also increase the respiration. Other carbohydrates and metabolic derivatives of carbohydrate either produce no significant change, or increase oxygen consumption slightly. Of the fatty acids, formic acid is not oxidized; propionic acid causes a small rise in oxygen uptake; but acetic acid and

Table 1. *Respiration of* Myco. phlei (*washed suspension*) *in the presence of various substrates*

(Medium: 3 ml. 0·033 M-phosphate buffer, pH 7·3, at 38°; gas: air; time: 2 hr.; Q_{O_2} = μl. O_2 consumed/mg. dry wt. bacteria/hr.)

Substrate	Conc. (M)	Q_{O_2} Substrate absent	Q_{O_2} Substrate present
Arabinose	0·02	−7·1	−8·1
Ribose	0·02	−5·9	−8·1
Glucose	0·02	−5·7	−25·3
Fructose	0·02	−6·5	−9·9
Fructosemonophosphate	0·01	−5·9	−8·5
Fructosediphosphate	0·01	−5·9	−8·5
Mannose	0·02	−7·7	−17·2
Galactose	0·02	−5·9	−6·7
Sucrose	0·02	−7·7	−7·6
Trehalose	0·02	−5·9	−13·2
Maltose	0·02	−5·9	−8·0
Lactose	0·02	−6·2	−6·3
Sorbitol	0·02	−6·7	−8·7
Glycerol	0·02	−5·9	−8·2
α-Glycerophosphate	0·01	−5·9	−7·2
β-Glycerophosphate	0·02	−5·9	−6·1
Gluconate	0·02	−7·5	−9·1
dl-Lactate	0·02	−5·9	−29·6
Pyruvate	0·02	−5·8	−11·6
Succinate	0·02	−5·7	−6·8
Fumarate	0·01	−6·2	−6·5
l(−)-Malate	0·02	−7·1	−11·6
Citrate	0·02	−7·7	−7·7
Formate	0·01	−6·2	−6·6
Acetate	0·02	−6·6	−11·1
Propionate	0·02	−6·1	−8·4
n-Butyrate	0·02	−6·1	−13·6
Crotonate	0·02	−5·9	−11·0
*iso*Valerate	0·02	−5·9	−7·7
n-Hexanoate	0·01	−5·9	−12·4
n-Octanoate	0·01	−5·9	−10·6
dl-β-Hydroxybutyrate	0·02	−6·7	−13·1
Acetoacetate	0·01	−8·6	−18·4

For 29 different cultures the mean Q_{O_2} in absence of added substrate was found to be −6·8 (standard deviation ±0·9).

higher homologues possessing an even number of C atoms roughly double the respiration rate.

Suspensions which have been heated to 100° for 5 min. have no oxygen uptake either in presence or absence of substrate.

Reduction of methylene blue

Methylene blue can function as hydrogen acceptor, although it is less effective than oxygen. Washed suspensions in phosphate buffer at pH 7·4 reduce methylene blue anaerobically, and the reduction time is diminished by addition of substrates (Table 2). The rather slow reduction times stand in

Table 2. *Anaerobic reduction of methylene blue by washed suspensions of* Myco. phlei

(Each evacuated Thunberg tube contained: 1 ml. washed aqueous suspension (2 mg. wet bacteria/ml.); 2 ml. 0·1 M-phosphate buffer, pH 7·4; 1 ml. methylene blue solution (1 in 5000); 0·5 ml. 0·2 M-substrate solution added from hollow stopper. Temp. 38°.)

Substrate	Reduction time (min.)
Nil	220
dl-Lactate	110
Acetate	180
Succinate	165
dl-β-Hydroxybutyrate	125
Glycerol	132
Glucose	105

the same relative order as the Q_{O_2} values of Table 1. The reduction time of the blank was not lengthened by aeration for several hours at 38°.

substrates are not oxidized by a common katabolic process. HCN (0·001 M) halves the 'blank' respiration and inhibits oxygen consumption in the presence of glucose by 84 %; under the same conditions respiration in the presence of lactate is inhibited by 39 % only. On the other hand, arsenite inhibits the respiration to a greater degree in the presence of lactate (35 %) than in the presence of glucose (12 %). Calculated on the basis of 'extra' oxygen uptake, the respective inhibitory effects of HCN and arsenite display an even greater contrast. This procedure, however, is dubious, because the influence of substrate on the spontaneous respiration is not easily determinable. Table 3 also shows that the oxidation of pyruvate and acetate is completely inhibited by arsenite.

Hydroxymalonate is known to inhibit the dehydrogenation of lactate by toluene-treated *Esch. coli* (Quastel & Wooldridge, 1928). Table 3 shows that *Myco. phlei* is able to oxidize hydroxymalonate. When hydroxymalonate and glucose are present together, the effect on oxygen uptake is additive; but the effect is competitive when lactate and hydroxymalonate are oxidized simultaneously, suggesting that the two α-hydroxy acids compete for the same enzyme.

Effects of crushing and of acetone treatment

The conclusion that *Myco. phlei* oxidizes glucose and lactate by essentially independent mechanisms is supported by the results of experiments on acetone powders and on extracts of crushed bacteria. Extracts were free from intact cells and coarser frag-

Table 3. *Influence of* HCN, *arsenite and hydroxymalonate on respiration of* Myco. phlei *in presence and absence of substrates*

(Washed suspension in 3 ml. 0·033 M-phosphate buffer, pH 7·3, at 38°; gas: air.)

Inhibitor	Washed bacteria ≡mg. dry wt./flask	Oxygen consumption (μl. in 1 hr.) in presence of: No substrate	0·01 M-glucose	0·01 M-*dl*-lactate	0·02 M-*dl*-lactate	0·01 M-pyruvate	0·02 M-pyruvate	0·02 M-acetate
Nil	9·6	74	279	261	—	—	—	—
HCN (0·001 M)	9·6	32	44	159	—	—	—	—
Nil	10·1	61	268	234	—	114	—	—
Arsenite (0·004 M)	10·1	53	238	151	—	54	—	—
Nil	9·7	46	—	—	251	—	70	67
Arsenite (0·0015 M)	9·7	47	—	—	215	—	49	46
Nil	4·4	20	116	88	—	—	—	—
Hydroxymalonate (0·066 M)	4·4	62	164	86	—	—	—	—

Effects of HCN, *arsenite and hydroxy-malonate*

Since lactate and glucose consume oxygen at approximately the same rate, it is possible that the former is an intermediate in the breakdown of the latter. This hypothesis is inconsistent with the experiments of Table 3 which show that these two

ments, but were slightly opalescent. The lactic acid oxidizing enzyme withstood both forms of treatment, whereas the systems that oxidize glucose, pyruvate and acetate were inactivated (Table 4).

There was a spontaneous oxygen uptake in the absence of added substrate with both preparations This blank was not affected significantly by glucose

pyruvate or acetate, but in the presence of *dl*-lactate there was a rapid oxygen consumption which began to fall off within an hour. Both the bacterial extract and the suspended acetone powder failed to consume oxygen after 5 min. heating in a water-bath at 100° regardless of the addition of substrate.

Table 4. *Oxygen consumption of crushed cellular extract and of acetone powder* (Myco. phlei)

(Bacterial extract: 0·5 ml. extract + 1·5 ml. 0·1 M-phosphate buffer; pH 7·3. Acetone powder: 15·6 mg. dry powder/3 ml. 0·03 M-phosphate buffer; pH 7·3; gas: air; temp. 38°.)

Substrate (0·033 M)	Oxygen uptake in 1 hr. (μl.) Bacterial extract	Acetone powder
Nil	40	38
Glucose	41	36
dl-Lactate	571	367
Pyruvate	56	42
Acetate	63	34
n-Butyrate	53	—

Aerobic and anaerobic CO_2 *production*

The aerobic CO_2 production of *Myco. phlei* suspended in bicarbonate buffer solution containing glucose is equal to the oxygen uptake (Table 5). This confirms the observation of Ebina & Nakamura (1937) that there is no aerobic glucolysis. Under anaerobic conditions (Table 5) there is a small CO_2 formation in the control which is not affected significantly by the presence of glucose, fructose or hexose diphosphate. Anaerobic CO_2 formation is increased slightly in the presence of pyruvate and acetoacetate. These experiments show that there is no glycolysis under the conditions used; and that the rate of anaerobic CO_2 production from ketonic acids is not commensurate with the rate of aerobic oxidation of these substrates (Table 1).

Table 5. *Aerobic and anaerobic* CO_2 *production by* Myco. phlei (*washed suspensions*)

Aerobic

(Two-vessel method of Warburg: 2·0 ml. of fluid in the large, 6·5 ml. in the small vessel. Medium: bicarbonate saline (NaCl, 0·16 M; $NaHCO_3$, 0·025 M) in equilibrium with gas mixture of 5% CO_2 and 95% O_2; pH 7·4; washed bacteria ≡ 4·6 mg. dry wt./cup; temp. 38°; time 70 min.)

	Oxygen uptake (μl.)	CO_2 formation (μl.)	CO_2/O_2
No substrate	35·5	33·5	0·94
Glucose (0·01 M)	89	90	1·01

Anaerobic

(Single conical Warburg vessels: 3 ml. bicarbonate Ringer solution (Krebs & Henseleit, 1932) in equilibrium with gas mixture of 5% CO_2 and 95% N_2. Phosphorus in the inseal; pH 7·4; washed bacteria ≡ 10·7 mg. dry wt./cup; temp. 38°; time 120 min.)

Substrate	$Q^{N_2}_{CO_2}$
Nil	0·6
Glucose (0·02 M)	0·7
Fructose (0·02 M)	0·7
Fructose-1,6-diphosphate (0·01 M)	1·0
Pyruvate (0·02 M)	1·9
Acetoacetate (0·01 M)	1·6

$Q^{N_2}_{CO_2}$ = μl. CO_2/mg. dry wt. bacteria/hr.

Table 6. *Residual bicarbonate after oxidation of glucose*

(Washed suspension of *Myco. phlei* ≡ 8·1 mg. dry wt./cup, in 2 ml. bicarbonate saline (NaCl, 0·16 M; $NaHCO_3$, 0·005 M) equilibrated with gas mixture containing 2% CO_2 and 98% O_2; pH 7·2; temp. 38°; time 2 hr.)

Substrate	Bicarbonate (μl. CO_2) Initial	Final
Nil	245	250
Glucose (0·01 M)	245	225

Measurement of residual bicarbonate (Table 6) reveals a diminution which may indicate the generation of small amounts of acid from glucose under aerobic conditions.

Utilization of glucose and lactate

Table 7 shows that both glucose and lactate disappear aerobically, but not anaerobically, in the presence of washed suspensions. 95% of the substrate was recovered from heated (100°) suspensions of bacteria. The deficit which occurred anaerobically is within the margin of experimental error.

The combustion of glucose and lactate is incomplete. Assuming that the endogenous respiration is unaffected by the metabolism of added substrate, the oxidation of 1 mmol. of either glucose or lactate consumes 1·3 mmol. of oxygen; but if the oxidation of substrate suppresses the endogenous respiration completely, the equivalence is 2·2 mmol. of oxygen. Although combustion is incomplete, the ratio CO_2/O_2 is approximately 1·0 with both substrates (Table 8).

Comparative rates of oxidation of glucose, lactate, pyruvate and acetate

The rates of consumption of oxygen by a washed suspension of *Myco. phlei* respiring in the presence of small amounts of the above substrates are shown in Fig. 1. The curves for pyruvate and acetate are almost linear, whereas those for glucose and lactate present an initial rapid phase before they become parallel with the pyruvate and acetate curves. At the point indicated by the arrow, glucose had disappeared from the suspension medium; addition of a further quantity of glucose beyond this point provoked a repetition of the curve.

Table 7. *Consumption of glucose and lactate by washed suspensions of* Myco. phlei

(Bacteria suspended in 3 ml. 0·033 M-phosphate buffer; pH 7·3; temp. 38°.)

	Washed bacteria (≡mg. dry wt.)	Time (min.)	Substrate added	Initial amount substrate (mg.)	Final amount substrate (mg.)	Oxygen uptake (μl.)	'Extra' oxygen consumed (μl.)	Substrate disappearing (mg.)
Anaerobic (gas: nitrogen; phosphorus in the inseal)	27·7	120	Nil	0·00	0·00	—	—	0·00
	27·7	120	Glucose	1·74	1·57	—	—	0·17
	34·0	120	Nil	0·00	0·00	—	—	0·00
	34·0	120	*dl*-Lactic acid	3·64	3·54	—	—	0·10
Aerobic (gas: air)	27·7	130	Nil	0·00	0·00	433	—	0·00
	27·7	130	Glucose	3·52	0·28	944	511	3·24
	34·0	180	Nil	0·00	0·00	361	—	0·00
	34·0	180	*dl*-Lactic acid	3·64	2·09	852	491	1·55

Table 8. O_2 *consumption and* CO_2 *production by washed suspensions of* Myco. phlei

(Medium: 3 ml. 0·03 M-phosphate buffer; pH 7·3 at 38°. Total CO_2 (free and bound) measured by the method of Warburg & Yabusoe (1924).)

Washed bacteria/cup (≡mg. dry wt.)	Time (min.)	Substrate	Oxygen uptake (μl.)	Total CO_2 (μl.)	CO_2/O_2
11·0	80	Nil	86	78	0·91
11·0	80	Glucose (0·01 M)	363	377	1·04
9·0	150	Nil	96	77	0·80
9·0	150	*dl*-Lactate (0·02 M)	327	336	1·03

The data of Table 7, obtained with larger amounts of substrate, represent the initial phase of oxidation of both glucose and lactate.

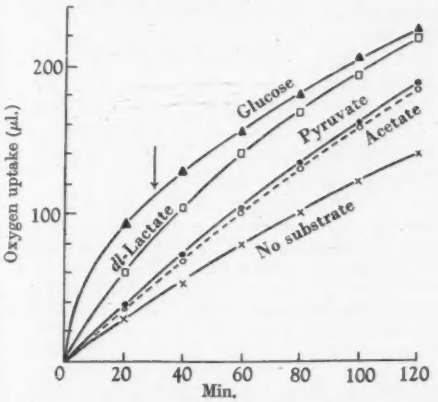


Fig. 1. Oxygen consumption of a washed suspension of *Myco. phlei* (≡26 mg. dry wt. bacteria/3 ml. 0·03 M-phosphate buffer, pH 7·4) at 38°. Gas: air. Initial quantity of substrate was 1/300 mmol. in each case. The arrow marks the point at which glucose had disappeared entirely from the suspension medium.

Products of oxidation

Products of incomplete combustion of glucose and lactate were sought, but in the case of glucose no product other than CO_2 has yet been identified. When the bacteria respire in the presence of arsenite, intermediate products of lactic acid breakdown can be detected. Traces of pyruvate (indicated by a faint olive green colour in the nitroprusside test applied to a filtrate which had been deproteinized with $\frac{1}{10}$ vol. 30% (w/v) trichloroacetic acid) were found together with larger amounts of acid volatile in steam which gave the blue lanthanum-iodine reaction (Table 9).

Having regard to the aerobic conditions, the CO_2/O_2 ratio (Table 8) and the oxygen uptake/mmol. lactic acid disappearing, it is almost certain that the volatile acid which reacts with the lanthanum-iodine reagent is acetic acid. Traces of this acid were found in the absence of arsenite (Table 9).

DISCUSSION

It is widely recognized that the acid-fast bacteria do not utilize carbohydrate anaerobically. Merrill (1930, 1931 *a*, *b*) has also shown that cleavage products of carbohydrate metabolism do not accumulate in synthetic media on which aerobic cultures have grown.

The experiments reported in this paper show that glucose and lactic acid are not oxidized completely by 'resting cells' of *Myco. phlei*. Clifton & Logan (1939), observing that the oxidation of a number of organic compounds by suspensions of *Esch. coli* was incomplete, suggested that a portion of the substrate was assimilated in the form of carbohydrate and the remainder oxidized to CO_2 and water

Table 9. *Products of oxidation of lactate*

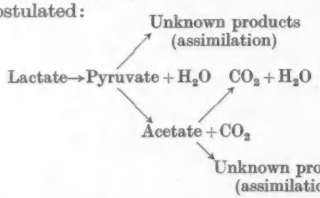
(Washed suspension of *Myco. phlei*; 75 mg./ml. distilled water; temp. 38°; gas: air; time 3·5 hr. 25 ml. stoppered conical flasks set up as follows)

Flask ...	1 (ml.)	2 (ml.)	3 (ml.)	4 (ml.)
Bacterial suspension	4·0	4·0	4·0	4·0
Phosphate buffer (0·1 M) pH 7·4	2·0	2·0	2·0	2·0
dl-Lactate (0·2 M)	—	1·0	—	1·0
Arsenite (0·05 M)	—	—	0·7	0·7
Distilled water	1·7	0·7	1·0	—
Lanthanum-iodine test	Neg.	±	Neg.	+ + + + +
Nitroprusside test	Neg.	Neg. or ±	Neg.	±

± = Trace.

A similar hypothesis could be applied to the glucose metabolism of *Myco. phlei* in the absence of contradictory evidence; but a different hypothesis is required to account for the incomplete combustion of lactic acid because extracellular products of intermediary metabolism have been detected.

The following sequence of aerobic reactions may be postulated:



Initial oxygen uptake is at least twice as fast in the presence of lactate as in the presence of pyruvate or acetate (Table 1 and Fig. 1). Although this rapid phase may represent the conversion of lactate to pyruvate and acetate, no more than a trace of either pyruvate or acetate can be found at any time during the oxidation.

When lactate is oxidized in the presence of arsenite (which completely inhibits the oxidation of added pyruvate or acetate (Table 3)) there is an accumulation of acetate but not of pyruvate (Table 9). Anaerobic dismutation of pyruvate, such as Krebs (1937) has described for the *Staph. aureus*, cannot account for the disappearance of pyruvate because anaerobic CO_2 formation is much too slow (Table 5). The disappearance of pyruvate formed as an intermediate in the breakdown of lactate—as distinct from added pyruvate—could be explained by a non-enzymic coupled oxidation involving H_2O_2 and leading to the formation of acetate. This hypothesis is consistent with the accumulation of acetate in the presence of arsenite and with the relative insensitiveness of lactate oxidation to HCN. It is supported by evidence obtained in a study of the system which oxidizes lactic acid in *Myco. phlei* (Edson, 1947).

Nevertheless, the hypothesis does not exclude the existence of an alternative route for the breakdown of lactic acid via pyruvic acid, since the oxidation of added pyruvate is enzymic and sensitive to arsenite. This alternative pathway may lead to processes of assimilation.

SUMMARY

1. Glucose and lactate are oxidized vigorously, but incompletely, by washed suspensions of *Myco. phlei*. They are not metabolized anaerobically.

2. Bacterial extracts and acetone powders oxidize lactate but not other substrates tested.

3. Comparative rates of oxidation of glucose, lactate, pyruvate and acetate have been determined.

4. Intermediary metabolites have been detected in the breakdown of lactate.

We are much indebted to Prof. H. A. Krebs for his kindness in reading the proofs.

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The Oxidation of Lactic Acid by *Mycobacterium phlei*

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Although lactic acid is utilized completely during the cultivation of *Mycobacterium phlei* (Stephenson & Whetham, 1922), it is oxidized incompletely by 'resting cells' (Edson & Hunter, 1947). Extracts of crushed cells and acetone-dried preparations, which retain power to oxidize lactate, lose the ability to oxidize pyruvate and acetate. Since an acetone powder will yield a soluble enzyme, this preparation has provided means of studying the mechanism of lactic acid breakdown.

METHODS

Manometric measurements. Oxygen uptake and CO_2 production were determined by the standard methods of Warburg. Substrates (acids as neutral sodium salts) were added after equilibration.

Analytical methods. Lactic acid was estimated by the method of Friedemann & Graeser (1933). In this procedure blank titrations of volatile bisulphite-binding material obtained from acetone powders never exceeded 0·3 ml. 0·005 *N*-I_2. Acetic acid was detected by the lanthanum-iodine test (see Edson & Hunter, 1947); and flavine-adenine-dinucleotide by the *d*-amino acid oxidase test of Warburg & Christian (1938).

Materials. *l*(+)-Lactic acid ($[\alpha]_D^{15°}$, Zn salt $= -8{\cdot}2°$) was obtained from the Pfanstiehl Chemical Co., U.S.A. *d*(−)-Lactic acid ($[\alpha]_D^{15°}$, Zn salt $= +8{\cdot}0°$) was prepared by resolution of *dl*-lactic acid (A.R. (British Drug Houses, Ltd.)) according to the morphine method of Irvine (1906). Catalase was crystallized by the method of Sumner & Dounce (1937) and dissolved in phosphate saline. Cozymase was prepared by the method of Williamson & Green (1940).

Acetone powder. The preparation and suspension of the powder have been described (Edson & Hunter, 1947). Acetone-treated bacilli are somewhat altered but retain their acid-fastness.

Preparation of a soluble enzyme. Because other methods of extraction proved unsatisfactory, 6·5 g. of dry powder were suspended in 40 ml. 0·02 M-phosphate buffer, pH 7·4, and ground for 4 hr. in the wet crushing mill of Booth & Green (1938). The gross debris was removed by centrifuging at 3500 r.p.m. for 0·5 hr. The supernatant liquid was brownish yellow and opalescent. Since high-speed centrifugal apparatus was not available, this fluid was diluted with an equal volume of 0·05 M-phosphate buffer and filtered through a bacteriological filter pad (Sterilmat G.S.). The filtrate was perfectly clear, bright yellow and non-fluorescent. It showed the Tyndall phenomenon and gave protein colour tests (biuret, Millon's) and a heat coagulum. The ability of the solution to oxidize lactic acid was not impaired after several weeks storage at 4°.

RESULTS

Rate of oxidation of dl-*lactate*

Table 1 shows the time course of the oxidation catalyzed by an acetone powder suspended in phosphate buffer. The initial high rate of oxygen consumption diminished progressively in successive 20 min. intervals until the oxidation ceased. In the last interval the increment is only slightly greater than that in the control without substrate. Heated suspensions have no oxygen uptake in the presence or absence of lactate. Table 1 also shows that pyruvate and acetate are not oxidized.

Table 1. *Oxygen consumption of acetone powder* (Myco. phlei) *in the presence of substrates*

(16 mg. acetone powder/3 ml. 0·06 M-phosphate buffer; pH 7·4; temp. 38°; gas: oxygen.)

	Oxygen uptake (μl.)			
Time (min.)	No substrate	*dl*-Lactate, 0·022 M	Pyruvate, 0·02 M	Acetate, 0·02 M
20	18	380	17	17
40	37	665	34	36
60	55	799	51	54
80	71	854	67	69
100	85	874	80	83
120	96	890	93	94

Effects of inhibitors

Spontaneous oxygen consumption in the controls is reduced by all the inhibitors used (Table 2); but the 'extra' oxygen uptake due to *dl*-lactate is not affected significantly by HCN, NaF or arsenite (0·01 M). Significant inhibitions were obtained with octyl alcohol (slight), NaN_3 and stronger arsenite (0·025 M). The insensitiveness of this lactate-oxidizing system to cyanide and its relative insensitiveness to azide distinguish it from lactic dehydrogenases which operate aerobically in conjunction with cytochrome systems, e.g. lactic dehydrogenase of yeast (Ogston & Green, 1935) and of the *Gonococcus* (Barron & Hastings, 1933).

Relationship between oxygen uptake and disappearance of lactic acid

When the extra oxygen consumption ceased, about half the *dl*-lactic acid which was added had disappeared, indicating that the enzyme may be

Table 2. *Effects of inhibitors on the oxidation of lactate*

(Medium: 3 ml. 0·033 M-phosphate buffer, pH 7·4; temp. 38°; gas: air. When HCN was used as inhibitor, the CO_2 absorbent was a mixture of KOH and KCN (Krebs, 1935).)

Weight of dried acetone powder/cup (mg.)	Time (min.)	Inhibitor	Oxygen uptake (μl.) Absence of lactate	Oxygen uptake (μl.) Presence of *dl*-lactate (0·02 M)	Inhibition of 'extra' O_2 uptake (%)
20·0	60	Nil	27	583	—
		NaN_3 (0·01 M)	15	392	32
		Octyl alcohol (saturated)	12	523	8
33·0	70	Nil	66	534	—
		HCN (0·01 M)	13	475	1
		Arsenite (0·01 M)	50	530	Nil
15·8	90	Nil	38	468	—
		NaF (0·01 M)	22	470	Nil
		Arsenite (0·025 M)	25	382	17

Table 3. *Oxidation of* dl-*lactate by acetone powder* (Myco. phlei)

(Medium: 3 ml. 0·033 M-phosphate buffer; pH 7·4; temp. 38°; gas: air. 31·6 mg. of acetone powder/cup. Time 80 min.)

dl-Lactic acid added (mg.)	Lactic acid formed	Lactic acid disappearing (mg.)	Oxygen uptake (μl.)	'Extra' O_2 consumed (μl.)	mmol. O_2 consumed/ mmol. lactic acid disappearing
Nil	Nil	Nil	74	—	—
5·38	—	2·47	635	561	0·92

(1) The experiment was stopped when the oxygen consumption was almost equal to that of the blank in a 10 min. interval.
(2) With the same suspension, which had been heated in a boiling water-bath for 10 min., there was no oxygen uptake and 5·36 mg. of lactic acid were recovered.

Table 4. *The oxidation of* l(+)- *and* d(−)-*lactate by acetone powder* (Myco. phlei)

(Medium: 3 ml. 0·033 M-phosphate buffer; pH 7·4; temp. 38°; gas: air.)

Weight of dried acetone powder/cup (mg.)	Time (min.)	Substrate	Amount of substrate added (mg.)	Amount of substrate disappearing (mg.)	Oxygen uptake (μl.)	'Extra' O_2 consumed	mmol. O_2 consumed/mmol. lactic acid disappearing
28·3	130	Nil	Nil	Nil	108	—	—
28·3	130	*l*(+)-Lactic acid	4·18	4·18	1098	990	0·95
28·3	130	*dl*-Lactic acid	5·40	2·57	722	614	0·96
18·6	120	Nil	Nil	Nil	40	—	—
18·6	120	*d*(−)-Lactic acid	4·40	Nil	41	1	—

(1) The experiments were stopped when the oxygen consumption was almost equal to that of the blank in a 10 min. interval.
(2) *d*(−)-Lactic acid was recovered completely (4·42 mg.).

specific for one optical isomer (Table 3). The volume of oxygen consumed agrees (within experimental limits) with the theoretical requirements of the ratio, 1 mmol. O_2/1 mmol. lactic acid. Control experiments have shown that the acetone powder does not form lactic acid spontaneously.

Stereochemical specificity of lactic acid oxidation

Simultaneous measurements of oxygen consumption and lactic acid disappearance show that the enzyme of acetone powder is entirely specific for the oxidation of *l*(+)-lactic acid (Table 4). The viable cells of *Myco. phlei* are, however, capable of oxidizing both stereoisomers (Table 5). In this case the rate

Table 5. *Oxidation of* l(+)- *and* d(−)-*lactate by viable organisms* (Myco. phlei)

(Medium: 3 ml. 0·033 M-phosphate buffer; pH 7·4; temp. 38°; gas: air; bacteria ≡14·2 mg. dry wt./cup.)

Substrate	Oxygen uptake 1 hr. (μl.)
Nil	93
dl-Lactate (0·02 M)	289
l(+)-Lactate (0·01 M)	293
d(−)-Lactate (0·01 M)	133
Acetate (0·01 M)	158
Pyruvate (0·01 M)	159

of oxidation of *l*(+)-lactate is nearly five times as fast as that of the *d*(−)-isomer, if it be assumed that the endogenous respiration is unaffected by the metabolism of the substrate. Both acetate and pyruvate are oxidized by the viable suspension at a much slower rate than *l*(+)-lactate.

The data of Table 4 also show that the oxidation of 1 mmol. of *l*(+)-lactic acid consumes approximately 1 mmol. of oxygen. The deficit is due in part to formation of a trace of pyruvic acid (see below).

Relationship between oxygen uptake and CO_2 production

The CO_2 production of an acetone powder suspended in phosphate buffer under aerobic conditions is increased by the presence of lactate, the ratio CO_2/O_2 becoming approximately 1·0 (Table 6). This fact and the data of Table 4 permit the reaction between lactic acid and oxygen to be formulated as follows:

$$CH_3.CHOH.COOH + O_2 = CH_3.COOH + CO_2 + H_2O. \quad (1)$$

Table 6. *Relationship between oxygen consumption and CO_2 production*

(Method of Warburg & Yabusoe (1924). 20 mg. acetone powder/cup. Medium; 3 ml. 0·03 M-phosphate buffer; pH 7·4; temp 38°; gas: air; time 2 hr.)

Substrate	Oxygen uptake (μl.)	Total CO_2 (free + bound) (μl.)	CO_2/O_2	'Extra' CO_2/O_2
Nil	−70	+34	0·49	—
dl-Lactate (0·022 M)	−592	+570	0·96	1·03

Products of oxidation

Acetic acid. In order to test the validity of the above equation the experiments were performed on a larger scale (7 ml. of buffered suspension containing 48 mg. of acetone powder and 22 mg. of sodium *dl*-lactate). This suspension was shaken aerobically at 38° for 4 hr., after which it was deproteinized by addition of 1 ml. 7% (w/v) metaphosphoric acid and filtered. The filtrate yielded an acid volatile in steam which gave an intense blue coloration in the lanthanum-iodine test. Control experiments in which lactate was omitted yielded traces of steam-volatile acid which gave no colour with the lanthanum-iodine reagents.

Although propionic and acetic acids react alike in the lanthanum-iodine test, the quantitative data exclude the possibility of a significant reduction of lactic acid.

Pyruvic acid. Attempts were made to detect the formation of pyruvic acid during the oxidation of lactic acid both in the presence and absence of arsenite. A trace of pyruvate can usually be found after the oxidation of 2–3 mg. of *l*(+)-lactate, the indication being a faint olive-green colour in the nitroprusside test. No such colour develops in control experiments without lactate.

The formation of a trace of pyruvate is confirmed by the results of micro-Clausen titrations (Clift & Cook, 1932). Reckoned as pyruvic acid, the bisulphite-binding substance amounts to 0·1 mg. or less.

Anaerobic dehydrogenation of lactic acid

An acetone powder prepared from *Myco. phlei* reduces methylene blue anaerobically. The reduction time is diminished considerably by addition of *dl*-lactate and pyruvic acid is formed (Table 7).

For the purpose of detecting pyruvic acid the contents of each tube were treated with 0·4 ml. 30% (w/v) trichloroacetic acid and filtered through a Whatman no. 40 paper (4·25 cm.). The filtrate was shaken to restore full colour to the residual dye and made alkaline with the minimum volume of NH_4OH (sp.gr. 0·88). Methylene blue was removed by adsorption on a pinch of activated charcoal. The clear, colourless filtrate was suitable for application of the nitroprusside test.

Methylene blue reduction was repeated on a larger scale. Acetone powder (0·7 g.) was suspended in 500 ml. of sterile 0·1 M-phosphate buffer, pH 7·4, in which 1·5 g. methylene-blue chloride had been dissolved. The suspension was distributed equally between two sterile flasks provided with ground-glass stopcocks. 10 ml. 2 M-*dl*-lactate were added to one; both flasks were then evacuated thoroughly and incubated at 38° for 6 days. Full aseptic precautions were observed throughout. At the end of the period of incubation the methylene blue was partially decolorized in the flask containing lactate.

The contents of each flask were cleared by addition of $\frac{1}{10}$ vol. 30% (w/v) trichloroacetic acid and subsequent filtration. The filtrate was made alkaline with NH_4OH and the residual methylene blue adsorbed on the minimum quantity of charcoal. The nitroprusside test was strongly positive (deep blue) with a 3 ml. sample from the flask to which lactate had been added, but negative with the control.

Table 7. *Anaerobic reduction of methylene blue by acetone powder from* Myco. phlei *in presence and absence of lactate*

(Each evacuated Thunberg tube contained 12·5 mg. acetone powder in 3 ml. 0·06 M-phosphate buffer, pH 7·3, and 0·5 ml. 0·5% methylene blue solution; temp. 38°; 0·3 ml. 0·2 M-*dl*-lactate solution added from hollow stopper at zero time. Conditions were aseptic.)

	Substrate	Reduction time (min.)	Pyruvic acid test
Unheated suspension	Nil	>300	Neg.
	dl-Lactate	160	Pos. (blue ring)
Heated suspension (previously heated to 100°)	Nil	>300	Neg.
	dl-Lactate	>300	Neg.

Each filtrate was acidified with HCl and mixed with $\frac{1}{5}$ vol. of a saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl. A yellow crystalline precipitate developed slowly in the cold in the filtrate which had given a positive nitroprusside test. This was collected by centrifugation, washed first with 0·2N-HCl and then with distilled water, and dried *in vacuo* (yield 33 mg.). The 2:4-dinitrophenylhydrazone of pyruvic acid was recrystallized from hot ethyl acetate. M.p. 215° (uncorr.).

Anaerobic CO_2 *formation by acetone powder*

It has been shown that the acetone powder catalyzes the dehydrogenation of lactic acid anaerobically with methylene blue as hydrogen acceptor, and that it does not oxidize added pyruvic acid under aerobic conditions. On this account it is necessary to explain the following facts: (1) pyruvic acid does not accumulate to any significant extent during aerobic oxidation of lactic acid even in the presence of arsenite; (2) the oxygen consumption is the amount required theoretically for conversion of lactic to acetic acid; and (3) acetic acid has been found.

The dilemma could be resolved if the acetone powder were able to catalyze anaerobic dismutation of pyruvate in the manner described by Krebs (1937). Table 8 shows that acetone powder forms CO_2 slowly under anaerobic conditions, but the rate is not affected significantly by addition of pyruvate, and therefore another explanation must be sought.

Table 8. *Anaerobic* CO_2 *production by acetone powder from* Myco. phlei

(10 mg. acetone powder/cup. Medium: 2 ml. bicarbonate saline (0·9% NaCl—0·025M-$NaHCO_3$); pH 7·3; temp. 38°; gas mixture: 5% CO_2 + 95% N_2. Phosphorus in the inseal.)

Substrate	CO_2 formation in 2 hr. (μl.)
Nil	27
Arsenite (0·02M)	24
Pyruvate (0·02M)	33
Pyruvate (0·02M) + arsenite (0·02M)	32

The possible role of hydrogen peroxide

The following sequence of events, involving an autoxidizable, heat-labile catalyst (X), is consistent with the over-all equation (1):

$$CH_3.CHOH.COOH + X = CH_3.CO.COOH + XH_2, \quad (2)$$

$$XH_2 + O_2 = X + H_2O_2, \quad (3)$$

$$CH_3.CO.COOH + H_2O_2 = CH_3.COOH + CO_2 + H_2O. \quad (4)$$

Reaction (4), which is quantitative, non-enzymic and almost instantaneous at 38°, was described by Holleman (1904). By means of the sensitive methods of Feigl (1937), it has not been possible to detect accumulation of H_2O_2 in aerobic experiments either in presence or absence of lactate. Moreover, the addition of pure catalase does not inhibit the oxidation of lactate by acetone powder (Table 9). It was also found, with the method of Stern (1932), that the acetone powder possessed a low catalase activity (kat. $f = 0·36$).

Table 9. *Effect of catalase on the oxidation of lactate*

Acetone powder

(7·1 mg. acetone powder suspended in 3 ml. 0·033M-phosphate buffer; pH 7·4; temp. 38°; gas: O_2. Catalase solution contained 500 μg. crystalline catalase/ml.)

Suspension (ml.)	3·0	3·0	3·0	3·0
Catalase solution (ml.)	—	0·5	—	0·5
dl-Lactate (0·2M) (ml.)	—	—	0·3	0·3
Distilled water (ml.)	0·8	0·3	0·5	—
O_2 uptake in 130 min. (μl.)	38	50	647	659

Soluble enzyme

(Dialyzed preparation dissolved in 0·033M-phosphate buffer; pH 7·4; temp. 38°; gas: air. Catalase solution contained 90 μg. crystalline catalase/ml.)

Enzyme solution (ml.)	1·5	1·5	1·5	1·5
Catalase solution (ml.)	—	0·2	—	0·2
dl-Lactate (0·2M) (ml.)	—	—	0·3	0·3
Distilled water (ml.)	0·5	0·3	0·2	—
O_2 uptake in 130 min. (μl.)	0	0	308	234

Soluble lactic enzyme of Myco. phlei

An extract of acetone powder free from cellular debris (see Methods) was fractionated according to the scheme of Fig. 1, partial separation of the enzyme being effected. The ability of each fraction to oxidize lactic acid is shown in Table 10. It will be seen that the activity of the lactic enzyme is not impaired by dialysis which is prolonged sufficiently to precipitate the bulk of the extracted protein. The active precipitate (fraction *E*) dissolved readily in phosphate buffer; the supernatant (fraction *F*) contained a small amount of inactive protein.

The original extract (*A*) gave a precipitate of yellow protein when brought to pH 4·5, leaving a yellowish supernatant. On re-solution in buffer this precipitate (fraction *B*) exhibited little activity. Most of the active protein (fraction *C*) was precipitated from the supernatant by $(NH_4)_2SO_4$ at pH 4·5. The residual protein (fraction *D*) was inactive. The operation of precipitating with $(NH_4)_2SO_4$ (0·3 saturation) at pH 4·5 was repeated twice with fraction *C*, the final solution in 23 ml. of phosphate buffer being pale yellow. At each repetition small amounts of inactive yellow protein were thrown down at pH 4·5 and removed prior to addition of $(NH_4)_2SO_4$.

Table 10. *Oxidation of lactate by fractions of the soluble enzyme preparation from* Myco. phlei

(Each manometric cup contained 1 ml. protein solution (see Fig. 1) in phosphate buffer; pH 7·4. Substrate: 0·3 ml. 0·2 M-*dl*-lactate; temp. 38°; gas: air.)

	Oxygen uptake in 1 hr. (μl.)
Original extract (*A*)	228
Fraction *E* (dialyzed enzyme)	209
Fraction *C* (precipitated and dialyzed)	210
Fraction *C* (twice reprecipitated and dialyzed)	215
Fraction *B*	12
Fraction *D*	Nil
Fraction *F*	Nil

(1) Controls without lactate had no oxygen uptake. (2) Addition of 0·2 ml. cozymase solution (5 mg./ml.) had no effect on oxygen uptake.

spontaneous oxygen consumption. The enzyme did not oxidize *dl*-alanine. Brief exposure to mineral acid (pH 2·0) at 0° caused inactivation.

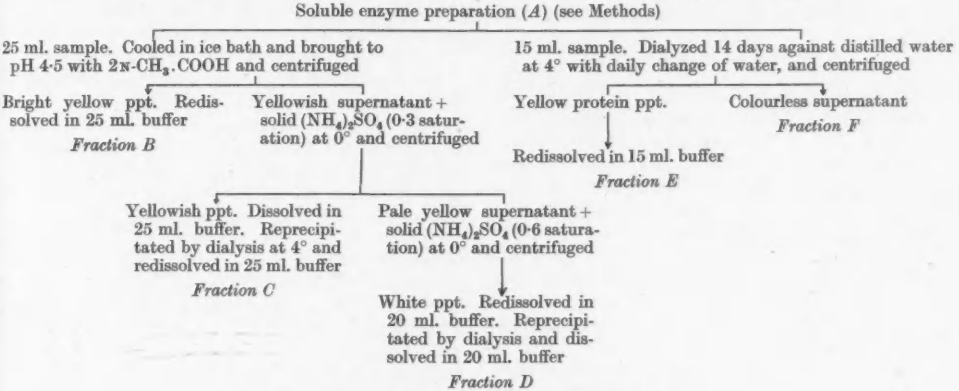
The behaviour of the original extract (*A*) and of the several fractions was unaffected by addition of cozymase. It may be noted that the lactic dehydrogenase of *Esch. coli* is also indifferent to the presence of cozymase (Still, 1941).

Addition of catalase partially inhibited the oxidation of lactate, an effect which was not observed with acetone powder (Table 9).

Occurrence of F.A.D. *in acetone powder and in soluble enzyme preparations*

When acetone powder was treated with N-HCl at room temperature, a clear yellow extract was

Fig. 1. *Fractionation of a soluble enzyme preparation from acetone powder from* Myco. phlei.


Soluble enzyme preparation (A) (see Methods)
25 ml. sample. Cooled in ice bath and brought to pH 4·5 with 2N-CH3.COOH and centrifuged
15 ml. sample. Dialyzed 14 days against distilled water at 4° with daily change of water, and centrifuged
Bright yellow ppt. Redissolved in 25 ml. buffer
Fraction B
Yellowish supernatant + solid (NH4)2SO4 (0·3 saturation) at 0° and centrifuged
Yellow protein ppt.
Colourless supernatant
Fraction F
Redissolved in 15 ml. buffer
Fraction E
Yellowish ppt. Dissolved in 25 ml. buffer. Reprecipitated by dialysis at 4° and redissolved in 25 ml. buffer
Fraction C
Pale yellow supernatant + solid (NH4)2SO4 (0·6 saturation) at 0° and centrifuged
White ppt. Redissolved in 20 ml. buffer. Reprecipitated by dialysis and dissolved in 20 ml. buffer
Fraction D

Fractions dissolved in 0·1 M-phosphate buffer; pH 7·4.

Table 11. *Occurrence of* F.A.D. *(flavine-adenine-dinucleotide) in extracts of* Myco. phlei

d-Amino-acid oxidase test. Each cup contained 2 ml. solution amino-acid oxidase protein (sheep kidney) in 0·1 M-phosphate buffer; pH 7·4; 0·2 ml. 0·5 M-*dl*-alanine in side-bulb; temp. 38°; gas: air.

	Vol. of extract (ml.)	Oxygen uptake (μl.) Before addition of alanine 30 min.	After addition of alanine 10 min.	20 min.	30 min.
HCl-extract of acetone powder, neutralized	0·5	Nil	46	97	149
Soluble enzyme fractions:					
Unheated, fraction *B*	0·5	Nil	1	4	6
Unheated, fraction *C*	0·5	Nil	3	6	8
Heated (100°), fraction *B*	0·5	Nil	26	58	92
Heated (100°), fraction *C*	0·5	Nil	20	46	72

(There was no oxygen uptake in controls from which amino-acid oxidase protein was omitted.)

Action of the soluble enzyme

Under both aerobic and anaerobic conditions the reactions of fractions *C* and *E* with lactic acid were qualitatively identical with those of an acetone powder, but in the absence of lactate there was no

obtained. This was bleached by a small amount of $Na_2S_2O_4$ and the colour restored by shaking with air. After neutralization the extract gave a positive result in the amino-acid oxidase test which is believed to be specific for flavine-adenine-dinucleotide (F.A.D.) (Table 11).

The presence of free F.A.D. has been demonstrated by the same test in some samples of the soluble enzyme preparation, but not in all. Combined F.A.D., however, was found consistently in the yellow fractions *B*, *C* and *E*, which gave positive results in the amino-acid oxidase test only after heating to 100° (Table 11). The yellow proteins were bleached by $Na_2S_2O_4$ and the colour restored by air. These facts indicate the presence of flavoprotein.

Influence of peptone on formation of the lactic enzyme

The ability of viable cells of *Myco. phlei* and of acetone-dried preparations to oxidize lactic acid was affected by the commercial peptone incorporated in the culture medium. Although the lactic enzyme was always present, the most active preparations were derived from organisms grown on broth containing Witte's peptone (Rostock). The depth of pigmentation appeared to run parallel with enzymic activity.

DISCUSSION

Stable enzyme preparations made from *Myco. phlei* oxidize *l*(+)-lactate to pyruvate anaerobically with methylene blue as hydrogen acceptor; aerobically, these preparations oxidize lactate to acetate and CO_2 without the addition of a hydrogen carrier. This distinguishes the enzyme from the lactic dehydrogenase of *Esch. coli* (Stephenson, 1928; Still, 1941) and of yeast (Dixon & Zerfas, 1939). Since the enzyme is insensitive to cyanide, it may be concluded that the aerobic oxidation is independent of a cytochrome system. Further, the preparations do not react with added pyruvate.

If it be assumed that pyruvate is an intermediary in the aerobic breakdown of lactate, the phenomena can be explained by a hypothesis of coupled oxidation outlined above in equations (2) to (4). If the assumption is invalid, the mechanism is different from any described hitherto.

Pursuing the assumption that pyruvate is an intermediary in the aerobic process, it is necessary to examine the nature of the heat-labile catalyst which could be either a specific autoxidizable flavoprotein (*l*-lactic acid oxidase) or a system composed of lactic dehydrogenase, codehydrogenase and an autoxidizable flavoprotein. The presence of a flavoprotein containing F.A.D. in the soluble preparation is consistent with both alternatives. It is to be noted that the bulk of the yellow protein precipitated at pH 4·5 in fraction *B* does not oxidize lactate.

Since activity is unaffected by dialysis and by repeated precipitation at pH 4·5, it is justifiable to conclude that a coenzyme is either unnecessary or non-dissociable. This distinguishes the lactic enzyme of *Myco. phlei* from the lactic dehydrogenase of animal tissues (Green & Brosteaux, 1936), but is consistent with the behaviour of a flavoprotein oxidase.

The results permit the following alternative formulations of the dehydrogenation of lactic acid:

(*A*)

$$CH_3.CHOH.COOH + Co = CH_3.CO.COOH + CoH_2$$

(Lactic dehydrogenase)

$$CoH_2 + F = FH_2 + Co$$

$$FH_2 + O_2 = F + H_2O_2,$$

or (*B*)

$$CH_3.CHOH.COOH + F' = CH_3.CO.COOH + F'H_2$$

$$F'H_2 + O_2 = F' + H_2O_2.$$

Co represents an unknown codehydrogenase; whilst *F* and *F'* represent autoxidizable flavoproteins. In both cases the scheme is completed by the spontaneous Holleman reaction:

$$CH_3.CO.COOH + H_2O_2 = CH_3.COOH + CO_2 + H_2O.$$

Since the Holleman reaction is rapid, it is unlikely to be a limiting factor in the conversion of lactate to acetate, and so the accumulation of pyruvate or H_2O_2 will not be expected. Under aerobic conditions a trace of pyruvate—but not of H_2O_2—has been detected after the action of acetone powder.

The mechanism postulated in (*B*) is analogous to the known behaviour of a purified *d*-amino-acid oxidase preparation (Negelein & Brömel, 1939), from which catalase has been removed. An acetone powder from *Myco. phlei* also possesses very little catalase. The addition of catalase to a suspension of the powder or to the soluble enzyme might be expected to diminish oxygen uptake and cause the accumulation of pyruvate. When this is done, the prediction is not fulfilled with acetone powder, although it is partially fulfilled with the soluble enzyme (Table 9). If the active surface at which H_2O_2 is nascent should be inaccessible to catalase, the main hypothesis is not falsified. A decisive test must await satisfactory purification of the enzyme.

It is evident from earlier observations (Edson & Hunter, 1947) that lactic acid can be broken down in the manner described in this paper by viable organisms which have been poisoned with HCN or arsenite. Normal cells oxidize pyruvate and acetate more slowly than lactate in reactions which must be partly assimilatory. Nevertheless, the same mechanism of lactic acid breakdown may apply to viable organisms.

SUMMARY

1. An acetone powder from *Myco. phlei* contains an enzyme which catalyses the reaction

$$CH_3.CHOH.COOH + O_2 = CH_3.COOH + CO_2 + H_2O.$$

The enzyme is specific for the oxidation of *l*(+)-lactate.

2. Anaerobically, lactate is oxidized to pyruvate.

3. The enzyme has been obtained in soluble form. Its properties are consistent with those of an autoxidizable flavoprotein.

I wish to thank Prof. H. A. Krebs for his kindness in reading the proof.

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The Mechanism of Phloridzin Glucosuria

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Glucose is reabsorbed in the kidney by the proximal tubule (Walker & Hudson, 1937). Lambrechts (1937) has shown that phloridzin is concentrated in the tubule at the same site. Although the *locus* of the action of phloridzin in producing glucosuria is thus identified as the proximal tubule, none of the theories which have been put forward explains to entire satisfaction the mechanism of the reabsorption of glucose and the manner of the action of phloridzin on this process. It is generally assumed that glucose is actively reabsorbed in the kidney by a process of phosphorylation. It has been suggested that phloridzin prevents glucose reabsorption by inhibiting glucose phosphorylation (Lundsgaard, 1933; Kalckar, 1936). This view is based mainly on the demonstration that phloridzin can inhibit glucose phosphorylation *in vitro*, e.g. by kidney slices, mince or extracts. The concentration of phloridzin necessary to inhibit phosphorylation of sugar is, however, definitely higher than the minimum concentration which brings about complete poisoning of reabsorption in the kidney (Lundsgaard, 1935). Kidneys of animals poisoned with phloridzin phosphorylated glucose at the same rate as normal kidneys.

Phloridzin exerts inhibiting effects on a wide variety of enzyme systems. Since such effects had not been observed except at a relatively high concentration of phloridzin, it was considered that they are irrelevant to the role of phloridzin in glucosuria. Re-examination of the validity of this view appeared desirable.

In a systematic test of the action of phloridzin on the different enzymes of carbohydrate desmolysis (Shapiro, 1939), the dehydrogenases of citric and pyruvic acid and certain systems which, by coupling with these dehydrogenases, generate high energy phosphate bonds, have been found to be highly susceptible to phloridzin inhibition. Marked inhibition of the susceptible systems was observed even at concentrations of phloridzin (0·0002–0·001 M) which are too low to affect glucose phosphorylation. It seems probable, therefore, that inhibition of glucose phosphorylation by phloridzin is but one of several secondary results of the action of phloridzin on reactions which generate high-energy phosphate bonds. The present paper summarizes data in proof of this statement. Experiments are also reported to show that the inhibitory effect of phloridzin on high-energy phosphate bond formation is manifested in the kidney of phloridzinized rats *in vivo* as well as *in vitro*.

METHODS

Rat kidneys were cooled on ice, the cortex separated and made into a fine mince with a scissors. When not otherwise stated, the mince (0·3–1·0 g.) was suspended in 5 vol. of Krebs's phosphate- or bicarbonate-Ringer solution (Krebs, 1933). Phloridzin (0·02 M) was dissolved in hot water and added to the reaction flasks to obtain the desired final phloridzin concentration.

Glucose was estimated according to Somogyi (1937). Lactic acid was determined by a modification of Friedemann's method according to Lieb & Zacherl (1932), inorganic phosphate according to Lohmann & Jendrassik (1926), creatine-phosphate as described by Potter (1945),

pyruvic acid according to Clift & Cook (1932) and citric acid according to Pucher, Sherman & Vickery (1936).

The tables contain representative results.

RESULTS

Inhibition of glucolysis by phloridzin

When incubated with kidney mince in phosphate-Ringer solution, glucose was utilized under aerobic as well as anaerobic conditions. Phloridzin at a concentration of 0·001 M inhibited glucose utilization in aerobic conditions but was relatively ineffective in the anaerobic experiments (Table 1). Concentrations of phloridzin below 0·001 M had no regular inhibitory effect on glucose utilization. A similar finding was indicated by analyses in which lactic acid formation was measured (Table 2).

Table 1. *Inhibition by phloridzin of glucose utilization in kidney mince*

1 g. kidney mince suspended in 5 ml. phosphate-Ringer solution containing 0·1% glucose, incubated for 1 hr. at 37°.

Exp. no.	Gas	mg. glucose utilized at phloridzin conc. 0	0·001 M	Inhibition (%)
1	Air	0·96	0·36	63
	Nitrogen	1·08	0·96	12
2	Air	0·81	0·48	44
	Nitrogen	1·00	0·96	4
3	Air	0·87	0·51	37
	Nitrogen	0·90	0·81	10

Table 2. *Inhibition by phloridzin of lactic acid formation in kidney mince*

Same mixture as above but with 0·2% glucose, incubated for 3 hr.

Exp. no.	Gas	mg. lactic acid formed at phloridzin conc. 0	0·001 M	Inhibition (%)
4	Air	1·38	0·94	32
	Nitrogen	1·72	1·58	8
5	Air	1·56	0·80	48
	Nitrogen	1·46	1·44	0
6	Air	2·20	1·24	43
	Nitrogen	1·72	1·60	7

Kidney mince was made weak in phosphate by dilution with bicarbonate-Ringer solution, 1 : 10 and 1 : 15. Phloridzin (0·001 M) failed to affect glucolysis by the diluted preparation (Table 3). Phloridzin inhibition reappeared, however, following addition of phosphate to the diluted preparation. When kidney mince was diluted with phosphate-Ringer solution no diminution in glucolysis susceptibility to 0·001 M-phloridzin occurred.

Table 3. *Dependence of the inhibition of glucose utilization on phosphate*

Dilution of kidney mince	Inhibition (%) by 0·001 M-phloridzin: In bicarbonate-Ringer solution	In phosphate-Ringer solution
1 g. in 5 ml.	41	41
1 g. in 5 ml.	28	37
1 g. in 10 ml.	11	36
1 g. in 10 ml.	0	27
1 g. in 15 ml.	5	30
1 g. in 15 ml.	0	28
1 g. in 15 ml. + 1 ml. phosphate-Ringer solution	18	—
1 g. in 15 ml.	0	—
1 g. in 15 ml. + 1 ml. phosphate-Ringer solution	27	—

Inhibition of dehydrogenase systems by phloridzin

The dependence of glucolysis inhibition by phloridzin on presence of oxygen and inorganic phosphate suggests that the point of attack of phloridzin is an oxidative process with which phosphorylation is coupled. In agreement with other authors it was found that phloridzin does not markedly reduce the respiration rate of the kidney. A number of individual respiratory systems was tested and several were found to be highly susceptible to phloridzin poisoning. The most marked inhibition was found in respect of the dehydrogenases of pyruvic and citric acids. These two dehydrogenases were completely inhibited by 0·001 M-phloridzin: in presence of phloridzin, addition of pyruvic and citric acid did not accelerate the decolorization of methylene blue.

Table 4. *Inhibition of dehydrogenases by phloridzin*

Kidney cortex mince, washed four times with M/15-phosphate buffer pH 5, suspended in 4 volumes of phosphate buffer at pH 7·4. 1 ml. of the suspension was introduced into a Thunberg tube and 0·1 ml. methylene blue (0·1%) and 0·2 ml. substrate (M/20) were added. The tubes were evacuated and incubated at 37°.

Exp. no.	Substrate	Time of decolorization (min.): Without phloridzin: No substrate	Without phloridzin: With substrate	With 0·001 M-phloridzin: No substrate	With 0·001 M-phloridzin: With substrate
1	Pyruvate	70	40	90	90
2	,,	40	15	55	55
3	,,	90	55	140	135
4	Citrate	41	14	75	56
5	,,	90	37	97	75
6	,,	90	27	140	135
7	Succinate	45	16	63	22
8	,,	41	6	75	6
9	,,	90	20	140	26

Succinic dehydrogenase, on the other hand, was not inhibited by the same concentration of phloridzin (Table 4).

Utilization of pyruvic acid and citric acid by kidney mince was found to be partially inhibited by 0·001 M-phloridzin. This suggests that not all the pathways of dissimilation are poisoned by phloridzin. Inhibition of pyruvic and citric acid utilization by phloridzin was nevertheless measurable even at concentrations of phloridzin as low as 0·0002 M (Table 5).

Table 5. *Inhibition by phloridzin of pyruvate utilization in kidney mince*

1 g. kidney mince incubated in 5 ml. phosphate-Ringer solution containing 0·1% sodium pyruvate for 2 hr. at 37°.

	Pyruvate utilized (mg.)			
	Without phloridzin	0·001 M-phloridzin	0·0002 M-phloridzin	0·0001 M-phloridzin
1	2·23	1·44	1·96	2·07
2	2·04	1·62	1·82	2·00
3	1·55	1·00	1·23	1·59

Table 6. *Inhibition by phloridzin of citrate utilization in kidney mince*

As in Table 5, but citrate (0·1%, Na salt) instead of pyruvate.

	Citrate utilized (mg.)			
	Without phloridzin	0·001 M-phloridzin	0·0002 M-phloridzin	0·0001 M-phloridzin
1	1·45	1·05	1·12	1·34
2	1·50	0·90	1·08	1·39

Table 7. *Inhibition of creatine-phosphate synthesis by phloridzin*

0·5 g. kidney mince suspended in 1·5 ml. of a mixture containing: 100 mg. creatine + 4 ml. water + 1 ml. KCl (0·5 M) + 1 ml. $MgCl_2$ (0·1 M) + 1 ml. NaF (0·25 M) + 1 ml. sodium phosphate buffer (0·2 M, pH 7·6), incubated for 20 min.

	Creatine-phosphate-P (μg.) synthesized with phloridzin						
	0	M/500	M/1000	M/1500	M/3000	M/4000	M/10000
1	35	4	9	11	14	18	32
2	40	5	5	9	—	12	22
3	38	—	3	—	6	8	—

Inhibition of coupled phosphorylation by phloridzin

As phosphorylation of adenylic acid is known to be coupled with pyruvate and citrate oxidation (Ochoa, 1941; Colowick, Welch & Cori, 1940), it is reasonable to consider that the inhibition of glucolysis and of glucose reabsorption in the kidney by phloridzin may be secondary to the primary effect of phloridzin on a susceptible dehydrogenase. Experiments were designed to study the influence of phloridzin on the phosphorylation of adenylic acid in the kidney. In the presence of phloridzin, permanent inhibition of phosphorylation was observed. The evidence of this finding was, however, inconclusive, as even in absence of phloridzin very low activity was found. To increase the rate of phosphorylation, a phosphate acceptor (creatine) was added to the kidney mince and high concentrations of fluoride and Mg^{++} were used to inhibit adenosine-pyrophosphatase (Potter, 1945).

Phosphorylation of creatine in kidney mince was completely abolished by 0·001 M and was markedly retarded by concentrations down to M/4000 phloridzin (Table 7). Small amounts of succinate (M/800) increased creatine phosphorylation without effecting phloridzin inhibition; concentrations of succinate of the order of 0·01 M, on the other hand, completely abolished phloridzin inhibition (Table 8).

Table 8. *Influence of succinate addition on the phloridzin inhibition of creatine-phosphate synthesis*

Conditions, other than succinate concentration, same as for Table 7.

	Creatine-phosphate-P (μg.) synthesized	
Addition	Without phloridzin	M/1000 phloridzin
No addition	23	6
M/800 succinate	54	10
M/100 succinate	59	57
No addition	28	5
M/800 succinate	43	8
M/100 succinate	41	37

Inhibition of coupled phosphorylation by injected phloridzin

Phloridzin, dissolved in propylene glycol, was injected into rats in a dosage of 25 mg./100 g. body weight. Control rats were injected with propylene glycol only. After half an hour, a second injection was given. The animals were sacrificed after an additional 15 min. Creatine-phosphate synthesis was then examined as before in the kidney tissue of these animals, M/800 succinate being added in order to raise the phosphorylation figures. The experimental and control groups numbered ten rats each, the other conditions were the same as in Table 7. 32 ± 2 μg. creatine-phosphate-P were synthesized by the control; 17 ± 3 μg. creatine-phosphate-P by the phloridzin injected group. It follows that a significant inhibition of the creatine-phosphate synthesis is caused by injection of phloridzin *in vivo*.

DISCUSSION

It has been shown in the present paper that pyruvic and citric acid oxidation in kidney is inhibited by a concentration of phloridzin as low as 0·0002 M. The

same phloridzin concentration also inhibits processes which generate high-energy phosphate bonds, and which are known to be coupled with these oxidations (Ochoa, 1941; Colowick *et al.* 1940). It seems necessary to assume that inhibition of susceptible dehydrogenase systems is the primary basis of phloridzin action in the kidney. This assumption is corroborated by the fact that phloridzin administered *in vivo* by injection is retained by the kidney in sufficient concentration to cause marked inhibition of creatine phosphorylation by the kidney. Lundsgaard (1935) showed in a 'pump-lung-kidney' preparation of a dog that complete poisoning of glucose reabsorption is ensured by 0·5–1·0 mg. of phloridzin/g. kidney. This dosage level corresponds to a concentration of M/3000–5000 in our test mixtures (1 g. kidney in 5 ml.). In this range of phloridzin concentration creatine phosphorylation is almost entirely inhibited. Glucose utilization as well as glucose phosphorylation (Kalckar, 1936) are inhibited to a similar degree only by 5–10 times as much phloridzin. Action of phloridzin on glucose utilization and phosphorylation may therefore be regarded as secondary to the inhibition by phloridzin of the production of high energy phosphate bonds.

These results are in agreement with the finding of Rapoport, Nelson, Guest & Mirsky (1941) who showed with the aid of labelled phosphate that the turnover of labile organic phosphate is reduced by phloridzin injection. Similar results were obtained by Kaplan & Greenberg (1944) with liver tissue. Our data suggest that the primary inhibitory action of phloridzin is exerted neither on phosphate transfer to adenylic acid nor on glucose phosphorylation by adenosine-pyrophosphate, as suggested by Beck (1942), but on oxidations with which phosphorylation of adenylic acid is coupled. This point is substantiated by the finding that the synthesis of high energy phosphate bonds proceeds normally in the presence of phloridzin if a substrate such as succinate, whose oxidation is not inhibited by phloridzin, is added to the system.

The assumption that phloridzin inactivates systems generating high energy phosphate bonds affords a plausible explanation of the finding that inhibition of reabsorption by phloridzin is not specifically limited to the case of glucose. Ellinger & Lambrechts (1937) observed failure of reabsorption of various dyes from the tubules of phloridzinized frogs. White (1940) has shown that secretion of diodrast is diminished by phloridzin. Evidently high energy phosphate bonds are important in kidney function for other purposes besides glucose reabsorption.

SUMMARY

1. Phloridzin at a concentration of 0·001 M inhibits glucose utilization and lactic acid formation by minced kidney cortex.

2. This inhibition is dependent upon the presence of both oxygen and inorganic phosphate.

3. Oxidation of pyruvic and citric acids, but not of succinate, is inhibited by concentrations of phloridzin as low as 0·0002 M.

4. The phosphorylation of creatine is inhibited by M/4000 phloridzin. The inhibition is abolished by M/100 succinate, but not by M/800 succinate which stimulates the phosphorylation in the absence of phloridzin.

5. Phloridzin given *in vivo* depresses creatine phosphorylation in minced kidney.

6. The results are explained by the assumption that phloridzin acts primarily on dehydrogenase systems that are coupled with phosphorylation. The bearing of these results on the question of the mechanism of phloridzin glucosuria is discussed.

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Further Studies of the Van den Bergh Reaction

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Azobilirubin production from sera of patients with regurgitation jaundice* (acute hepatitis and obstructive jaundice) was found under the conditions of the modified Rappaport & Eichhorn method (1943) to be maximal within 30 min. in both the direct and the indirect reaction (Gray & Whidborne, 1946). Sera from the few cases of retention (haemolytic) jaundice studied gave two forms of azobilirubin production-time curve; in one, azobilirubin production in the direct reaction was low and delayed, while in the other it was high and more rapid, requiring only 0·5 hr. to reach a maximum. In both, azobilirubin production in the indirect reaction was maximal only after about 5 hr. The present paper presents further observations of azobilirubin production from sera from patients with regurgitation jaundice and describes the results given by a larger series of sera from patients with retention jaundice. These last results suggested that differences in the diazo reaction of bilirubin-containing sera might be due to the presence in some of a substance or substances able to catalyze the formation of azobilirubin. It therefore appeared to be of value to investigate the diazo reaction of bilirubin when added to sera likely to contain pathological constituents or physiological constituents in excess.

MATERIALS

The nature of the material investigated is summarized together with the corresponding results in Table 1. Preliminary investigations showed no difference in the behaviour of sera or plasma in the diazo reaction, and since greater yields of plasma than of serum can be obtained from samples of blood, many of the experiments have been carried out on plasma. In the account which follows, the term serum will be used for the material investigated irrespective of whether it was in fact serum, plasma or pleural fluid. All materials contained at least 1·5 mg. bilirubin/100 ml. The nature of the sera to which bilirubin was added is indicated together with the results given by such mixtures in Table 3.

METHODS

The rates of azobilirubin production were determined by the modified Rappaport & Eichhorn procedure previously described (Gray & Whidborne, 1946) but using a Spekker photoelectric absorptiometer and 0·5, 1·0 or 2·0 cm. cells according to the amount of bilirubin present in the serum. With sera from patients with retention jaundice, azobilirubin production in the direct reaction (i.e. in the absence of caffeine and citric acid) usually attained its maximum in less than 5 hr., but a few sera failed to give a constant value within this time. They often became opalescent on further standing, and the reading for the direct reaction was therefore never followed for longer than 5 hr. More rarely, the azobilirubin solution would fade before the 5 hr. had elapsed and the extent of the direct reaction has then been calculated from the maximum reading of the optical density. In the indirect reaction (i.e. in the presence of caffeine and citric acid), azobilirubin production was usually maximal within 6 hr. but occasionally not until 18–24 hr. when readings were continued for this length of time.

From these readings we calculated the direct-indirect quotient (D.I.Q.) defined as:

$$\frac{\text{final amount of azobilirubin formed in the direct reaction}}{\text{final amount of azobilirubin formed in the indirect reaction}} \times 100.$$

For simplicity of presentation and discussion we have also introduced two additional terms which it is necessary to define:

(1) The direct-quotient $(\text{D.Q.})_t = \dfrac{\text{amount of azobilirubin formed in the direct reaction at time } t}{\text{final amount of azobilirubin formed in the indirect reaction}} \times 100.$

Graphs of $(\text{D.Q.})_t$ plotted against time therefore record the course of the direct reaction.

(2) The indirect-quotient $(\text{I.Q.})_t = \dfrac{\text{amount of azobilirubin formed in the indirect reaction at time } t}{\text{final amount of azobilirubin formed in the indirect reaction}} \times 100.$

Graphs of $(\text{I.Q.})_t$ plotted against time therefore record the course of the indirect reaction.

* In this article the nomenclature used will be that of Rich (1930) who classified jaundice into retention and regurgitation types. Retention jaundice corresponds to haemolytic jaundice but regurgitation jaundice is subdivided into mechanical and parenchymal types corresponding to obstructive and hepatogenous jaundice respectively.

In many experiments, azobilirubin production was also studied in the presence of the same buffers as ordinarily used but from which urea had been omitted. Urinary urobilin and urobilinogen were measured in a few cases by the method of Watson (1936). Bilirubin was added to serum by dissolving 3 mg. of pure bilirubin in 3 ml. of 0·2N-sodium carbonate at 80°. After cooling, 1 ml. of this solution was added to 9 ml. of serum. The bilirubin content of each serum was determined before and after the addition of the bilirubin, and the $(D.Q.)_t$ and $(I.Q.)_t$ followed at the usual intervals of time in the presence and absence of urea. Comparison solutions were prepared in which water replaced serum.

both the direct and indirect reactions occasionally requires between 0·5 and 1 hr. to reach its maximum instead of less than 0·5 hr. as previously reported but over 90 % of the maximum value has always been attained within 0·5 hr.

(*b*) *Retention jaundice.* Only two sera and two pleural fluids were found in which the D.I.Q. as measured by the Rappaport & Eichhorn method was less than 50. One serum was from a patient who had been transfused with haemolyzed blood, the other from a patient with non-familial haemolytic jaundice. The $(D.Q.)_t$ time and $(I.Q.)_t$ time curves given by these materials are presented in Figs. 1*a*, *b*, *c* and *d*.

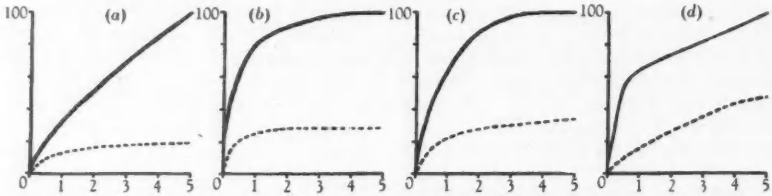

Fig. 1. Four observed D.Q.$_t$ time and I.Q.$_t$ time curves. D.I.Q. less than 50 with each serum. (*a*) Serum from patient transfused with haemolyzed blood. (*b*) Serum from patient with non-familial haemolytic jaundice. (*c*) Pleural fluid. (*d*) Pleural fluid.

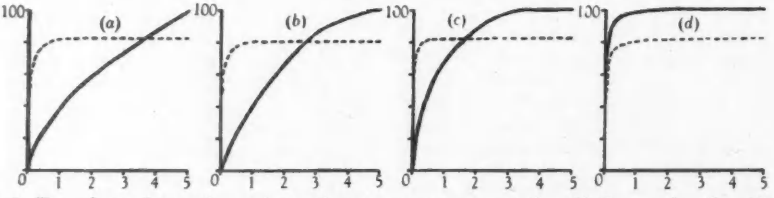

Fig. 2. Four observed D.Q.$_t$ time and I.Q.$_t$ time curves. D.I.Q. greater than 50, $T_{75\ \%\ D.Q.}$ less than 0·5 hr.

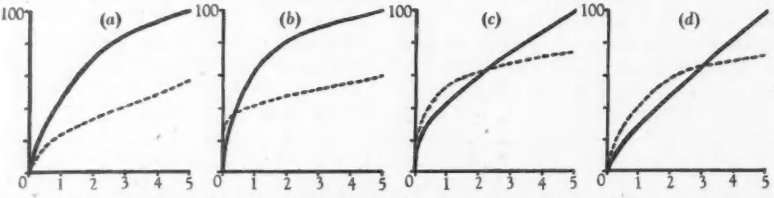

Fig. 3. Four observed D.Q.$_t$ time and I.Q.$_t$ time curves. D.I.Q. greater than 50, $T_{75\ \%\ D.Q.}$ greater than 0·5 hr. (*a*) and (*b*) Sera from patient with non-familial haemolytic jaundice. (*c*) Pleural fluid. (*d*) Serum from patient with constitutional non-haemolytic jaundice.

Figs. 1–3. Ordinates = $(D.Q.)_t$ or $(I.Q.)_t$. Abscissae = time in hours. D.Q.$_t$ time curve ------. I.Q.$_t$ time curve ——.

RESULTS

Form of the $(D.Q.)_t$ *and* $(I.Q.)_t$ *time curves*

(*a*) *Regurgitation jaundice.* Further study of cases of regurgitation jaundice has shown that when the bilirubinaemia is very high (i.e. above 15 mg./100 ml.) or when the jaundice has persisted for longer than 3 weeks the azobilirubin production in

All remaining materials gave D.I.Q. values between 50 and 100 but no correlation could be found between the value of the D.I.Q. and the type of fluid examined. In all but four (see below), the $(D.Q.)_t$ time curve was steep initially, $(D.Q.)_t$ reaching 75 % of its final value within 0·5 hr., the curves then flattening gradually as the $(D.Q.)_t$ approached its maximum and final value. Such curves resemble the $(D.Q.)_t$ time curves

observed with sera from patients with regurgitation jaundice. The $(I.Q.)_t$ curves have varied considerably ranging from a uniformly gradual slope (Fig. 2*a*) to the steep beginning and gradual termination of Fig. 2*d*, with the intermediate forms represented in Fig. 2*b* and *c*. The curves have been arbitrarily classified as types 2*a*, *b*, *c* and *d* according to whether the time ($T_{75\%\,I.Q.}$) taken for the $(I.Q.)_t$ to reach 75% of its final value has been 3, 1·5–3, 0·5–1·5 or less than 0·5 hr.

The four exceptional materials referred to above in which the $(D.Q.)_t$ failed to reach 75% of its final value within 0·5 hr., although the D.I.Q. value was greater than 50, included sera from two cases of non-familial haemolytic jaundice, one of constitutional non-haemolytic jaundice and one pleural fluid. The four pairs of curves obtained are shown in Fig. 3*a*, *b*, *c* and *d*. In each there was delay in the rate of formation of azobilirubin in the direct reaction as well as in the indirect reaction.

Sera have been examined from seven cases of haemolytic jaundice following the transfusion of incompatible blood. At the height of the jaundice, i.e. 1 or 2 days after the transfusion, curves of the type 2*a* or *b* were obtained, but in the course of a few days the form of curve changed to type 2*d* resembling that obtained in regurgitation jaundice. Similar changes in the form of curve have been observed in two cases of severe haemolytic jaundice of very acute onset. The detailed distribution of the types of curve yielded by the various types of case is summarized in Table 1.

rubin production is also retarded. With sera from patients with regurgitation jaundice, the decrease in D.I.Q. usually amounted to only 5% or less and the general form of curve was unaltered. One serum, however, showed a difference of 12%. On the other hand, with sera from patients with retention jaundice giving forms of curve other than of type 2*d*, omission of urea greatly lowers the D.I.Q. and considerably retards the rate of azobilirubin production so that the general form of the $(D.Q.)_t$ time curve is profoundly altered. The final concentration of urea in the direct reaction is 18·7 g./100 ml. which of course far exceeds the concentrations likely to be encountered in pathological sera. It has not been possible to compare accurately the effect of dilution by the phosphate buffer on the course of the direct reaction since when the diazo reaction was performed on undiluted sera an opalescence rapidly appeared preventing accurate photoelectric measurement of the azobilirubin produced under these conditions. As far as can be judged by eye the course of the direct reaction is not significantly altered by such dilution.

The diazo reaction of bilirubin added to serum

Some difficulty was encountered in the investigation of the solution of bilirubin in water since on the addition of the diazo reagent a brown precipitate appeared which in the case of the direct reaction settled to the bottom of the absorption cell and could not be induced to pass back into solution. A similar precipitate was obtained in the indirect reaction but this gradually redissolved during the experiment. It is therefore not surprising that the D.I.Q. amounted to only 27 and that the graphs $(D.Q.)_t$ and $(I.Q.)_t$ plotted against time were similar to curve 1*d*.

Table 1. *Materials investigated and their diazo reactions*

Type of jaundice	No. of cases		No. of sera		Type of curve 1	2*a*	2*b*	2*c*	2*d*	3
Infective jaundice	19	33	23	42	0	0	0	0	23	0
Obstructive jaundice	8		9		0	0	0	0	9	0
Chronic hepatitis	4		7		0	0	0	0	7	0
Homologous serum jaundice	2		3		0	0	0	0	3	0
Transfusion reactions	7	33	22	65	1	3	4	4	10	0
Familial haemolytic jaundice	11		11		0	1	1	8	1	0
Non-familial jaundice	9		18		1	2	5	6	2	2
Miscellaneous cases with definite haemolytic element but of doubtful origin	5		13		0	2	2	1	8	0
Constitutional non-haemolytic jaundice	1		1		0	0	0	0	0	1
Pleural fluids	11		12		2	2	3	1	3	1

Table 2. *Effect of urea (24 g./100 ml.) in the buffer used for the direct Van den Bergh reaction*

Type of jaundice	No. of cases	D.I.Q. Urea present	D.I.Q. Urea absent
Regurgitation	12	77±1·9 (68–91)	75±1·6 (68–84)
Retention	11	78±4·8 (51–100)	35±5·6 (8–78)

Effect of urea on the direct reaction

Table 2 shows that the omission of urea from the direct buffer decreased the D.I.Q. The rate of azobili-

Table 3. *Results given by bilirubin added to sera*

Nature of serum	Total bilirubin of serum (mg./100 ml.) Before addition of bilirubin	After addition of bilirubin	D.I.Q. with urea	$T_{75\ \%\ D.Q.}$ with urea (hr.)	D.I.Q. without urea	$T_{75\ \%\ D.Q.}$ without urea (hr.)	$T_{75\ \%\ I.Q.}$ (hr.)
Normal	1·0	11·2	74	1·0	21	2·8	4·1
Normal	0·8	10·8	72	1·5	19	2·0	5·0
Normal	0·4	11·0	67	1·25	20	2·0	4·7
Normal	0·4	11·7	84	1·0	17	2·0	5·5
Normal	0·4	11·7	80	0·8	20	2·0	5·5
Hypercholesterolaemia (nephritis)	0·4	10·6	66	1·9	15	2·0	2·8
Hypercholesterolaemia (nephritis)	0·4	9·8	84	0·5	18	2·0	5·0
Hypercholesterolaemia (myxoedema)	0·4	9·9	85	0·2	24	4·5	5·2
Haemolytic jaundice	3·3	14·8	80	0·1	12	2·0	4·75
Recovery stage of acute hepatitis	2·2	11·8	80	1·25	19	0·5	6
Acute hepatitis	9·8 (D.I.Q. 70)	20·2	57	0·75	41	0·3	2·5
Obstructive jaundice	10·2 (D.I.Q. 84)	20·1	66	0·5	40	0·25	2·25
Chronic liver damage (cirrhosis)	0·4	10·4	81	0·1	15	1·0	3·3
Chronic liver damage (cirrhosis)	3·7	14·2	80	0·1	22	0·25	2·6
Chronic liver damage (cirrhosis)	0·5	12·0	80	0·1	21	0·25	4·5

With bilirubin added to serum, no precipitation occurred and the results given by such mixtures are summarized in Table 3. The D.I.Q. was always greater in the presence of urea than in its absence, and except for the two experiments in which bilirubin was added to serum already containing an appreciable excess of bilirubin, the differences in D.I.Q. in the presence and absence of urea were of the order of those observed in retention jaundice. In all experiments, the $(I.Q.)_t$ time curves were of the same form, maximum azobilirubin formation requiring from 3 to 8 hr.

$(D.Q.)_t$ *curves in the presence of urea.* The $(D.Q.)_t$ time curves given by bilirubin added to normal sera, to serum from a patient recovering from acute hepatitis and to the serum from one of the patients with hypercholesterolaemia, were of gentle slope and reached their maxima within 1·1 and 2·7 hr. Bilirubin added to the two other hypercholesterolaemic sera gave a slightly accelerated direct reaction. Bilirubin added to sera from three patients with chronic liver damage showed in each case a marked acceleration of the direct reaction comparable with that represented in Fig. 2*d*.

$(D.Q.)_t$ *curves in the absence of urea.* Bilirubin added to the normal sera, to all three hypercholesterolaemic sera and to the serum from the patient with haemolytic jaundice gave the delayed reactions of type 1*d*. Addition of bilirubin to sera from two of the three patients with chronic liver damage caused markedly accelerated reactions, but on addition to the serum of the third patient and to that of the recovering hepatitis patient, the acceleration of the direct reaction, though still present was less striking.

The reactions of bilirubin added to the two sera already containing appreciable amounts of bilirubin are of particular interest (see below) and the $(D.Q.)_t$ and $(I.Q.)_t$ time curves have been presented in Fig. 4.

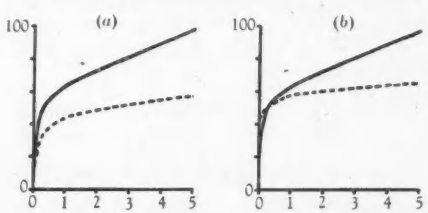

Fig. 4. Observed $D.Q._t$ time curves of bilirubin added to two jaundiced sera already containing approximately 10 mg./100 ml. of bilirubin.

Ordinates = $(D.Q.)_t$ or $(I.Q.)_t$. Abscissae = time in hours. $D.Q._t$ time curve - - - - -. $I.Q._t$ time curve ——.

DISCUSSION

The reasons for the differences in character of the Van den Bergh reaction as encountered in clinical medicine have long been obscure. Any theory based on one type of experiment has soon been succeeded by another supported equally well by experimental evidence of a different kind. Van den Bergh (1918) originally believed that in serum containing bilirubin giving a negative direct reaction the bilirubin was bound to the serum protein and that the addition of ethanol was therefore necessary to liberate bilirubin from the protein complex. Bennhold (1932) and Pedersen & Waldenström (1937),

however, showed by electrophoretic and ultracentrifuge studies that the bilirubin is bound to the plasma albumin whether the direct reaction is positive or negative. Coolidge (1940) confirmed this and postulated that the indirectly reacting bilirubin was chemically bound by valency bonds, whereas the direct reacting type is simply absorbed loosely. Others have postulated two different forms of bilirubin responsible for the two types of reaction (Fowweather, 1932; Davis & Sheard, 1937; Heilbrun & Hubbard, 1940), while the remaining possibility that the direct reaction might be due to the presence of catalysts in the serum itself has not been without its supporters (Acil & Golgruber, 1932). The present work lends some support to this last theory.

The diazo reaction in retention jaundice

In the early stages of these investigations the only types of direct reaction given by sera from patients with retention jaundice were those represented by Figs. 1*a* and 2*b* and the possibility was considered that the second type of reaction was caused by the presence in the serum of two forms of bilirubin one of which gave $(D.Q.)_t$ and $(I.Q.)_t$ time curves similar to Fig. 1*a* and the other the curves typical of regurgitation jaundice (Fig. 2*d*). Subsequent experience has shown that in retention jaundice many types of reaction occur varying imperceptibly from one extreme to the other.

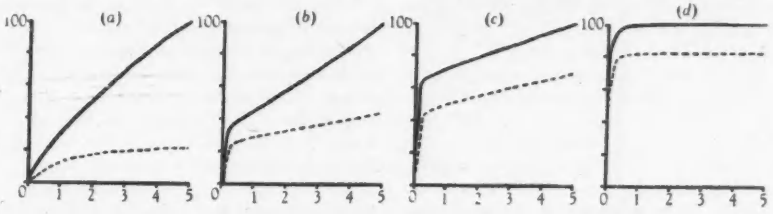

Fig. 5. Theoretical $D.Q._t$ time and $I.Q._t$ time curves expected from materials giving curves of types 1*a* and 2*d*. (*a*) 100% 1*a*. (*b*) 66% 1*a*; 33% 2*d*. (*c*) 33% 1*a*; 66% 2*d*. (*d*) 100% 2*d*.
N.B. Ordinates = $(D.Q.)_t$ or $(I.Q.)_t$. Abscissae = time in hours. $D.Q._t$ time curve - - - - - - . $I.Q._t$ time curve ——.

If we suppose that there are two types of bilirubin in serum one of which gives the type of reaction 1*a* and the other the type 2*d*, we should expect mixtures of the two to give the series of summation curves represented in Fig. 5. Although one of these (5*b*) somewhat resembles the observed curves of type 3*b*, simple summation cannot account for the other observed curves unless the existence of the two forms of bilirubin in the same serum grossly modifies their individual behaviour under the conditions of the test, which appears unlikely. It is more probable that the gradation in form of the curve is due to the presence of some factor exerting its effect in varying degrees either by direct catalysis of the reaction or by interference with some inhibiting factor present in the blood stream and cause some degree of change in the reaction. This factor would appear to act most effectively in regurgitation jaundice in which direct and indirect reactions of the type 2*d* always occur. A similar series of reactions may be given by haemorrhagic pleural effusions into which the factor may also pass.

The effect of urea on the direct reaction

The difference between the measurements of direct bilirubin by the Malloy & Evelyn (1937) method and by that of Rappaport & Eichhorn (1943) was ascribed in our earlier paper to the difference in pH of the two reaction mixtures but it is now seen that the presence of urea in the latter method also is an

in sera giving reactions of type 1*a*. This factor appears to accelerate the reaction more readily in the absence of caffeine and citric acid than it does in their presence (i.e. in direct than in indirect reaction), but presumably as its effect becomes more marked the reaction in the presence of these reagents also becomes accelerated to varying degrees, thus accounting for the reactions seen in Fig. 2. With its maximum effect both $(D.Q.)_t$ and $(I.Q.)_t$ time curves are of the forms seen in regurgitation jaundice. Not only rate but also extent of reaction appears to be accelerated by this hypothetical factor (compare D.I.Q. in Figs. 1 and 2). It has long been considered by many workers (Watson, 1937) that haemolytic jaundice is rarely, if ever, purely haemolytic in origin, but that there is usually an associated impairment of hepatic function. As far as is known this impairment of function is usually confined to the function of pigment excretion and it appeared of value, therefore, to determine whether or not change in form of the diazo reaction was accompanied by an increased excretion of urinary urobilin and urobilinogen. The few determinations of these pigments we have been able to make have been consistent with this possibility. It therefore appears possible that in pure retention jaundice with intact hepatic function the direct reaction may follow the course depicted in Fig. 1*a*. If there is associated liver damage then the unknown factor may accumulate

important factor. The effect of the presence of urea on the direct reaction given by sera from patients with regurgitation jaundice is minimal, and the application of our earlier results (Gray & Whidborne, 1946) to the interpretation of the direct reaction in this condition therefore requires no revision. The profound effect of urea on the direct reaction given by sera from patients with retention jaundice indicates that in this condition the results obtained with the Rappaport & Eichhorn technique will not necessarily correspond with the course of the ordinary direct Van den Bergh reaction as usually performed. For this reason the direct reaction of many of the materials used has been investigated both in the presence and the absence of urea, since it is believed that the direct reaction under the latter condition is more representative of the ordinary reaction. The differences observed in the direct reaction given by various haemolytic sera in the presence of urea, however, were sufficiently striking to be worthy of the consideration accorded them in the previous section.

The diazo reaction of bilirubin added to serum

Although in these experiments with bilirubin added to sera, the direct reaction in the absence of urea more closely resembles the customary Van den Bergh reaction, the results obtained in the presence of urea have also been presented in order that they may be considered relative to the earlier work on naturally occurring sera without added bilirubin in the presence of a buffer containing urea.

The addition of bilirubin to normal serum results in a mixture which gives a diazo reaction similar to that given by sera from some patients with retention jaundice, i.e. those giving reactions of type 3. Similar treatment of certain pathological sera as detailed below resulted in accelerated direct diazo reactions both in the presence and in the absence of urea. It is important to note that in the absence of urea neither was the D.I.Q. increased nor was the indirect reaction accelerated in the way characteristic of regurgitation jaundice.

Hypercholesterolaemia. Both ester and free cholesterol were increased in the sera used. On two occasions, there was some acceleration of the direct reaction in the presence of urea, but no effect in its absence, nor on the course of the indirect reaction. Increased cholesterol content cannot therefore be the cause *per se* of the accelerated direct reaction in regurgitation jaundice.

Haemolytic jaundice. The marked acceleration of the direct reaction in the presence of urea of bilirubin added to serum from the patient with haemolytic jaundice may possibly be related to the fact that this serum originally gave a similar direct reaction.

Acute hepatitis. Bilirubin added to serum from the patient recovering from acute hepatitis showed acceleration of the direct reaction in the absence of urea but paradoxically not in its presence. No accleration of the indirect reaction resulted, so that if the characteristic nature of the diazo reaction in acute hepatitis is due to the accumulation in the blood of an accelerating factor, this factor must disappear rapidly with the return of the blood bilirubin to normal levels.

Chronic liver damage. Addition of bilirubin to sera of patients with chronic liver damage resulted in considerably accelerated diazo reactions in the presence of urea and in two cases in the absence of urea, but again the $(\text{I.Q.})_t$ time curves were unaltered.

These experiments thus suggest that the factor causing the characteristic Van den Bergh reaction in regurgitation jaundice must be fairly specifically retained in the blood in this condition.

The results obtained with bilirubin added to sera already containing appreciable amounts of bilirubin require consideration in some detail. Fig. 4 shows that the $(\text{D.Q.})_t$ and $(\text{I.Q.})_t$ time curves resemble closely the theoretical curves obtained by a mixture of equal amounts of bilirubin giving two different types of reaction (i.e. curves midway between those represented in Fig. 5*b* and *c*). These results alone in the present series of investigations support the view that there may be two different forms of bilirubin. However, the D.I.Q. of the mixture was less than the average of the values for the two materials.

It is not easy to correlate these results with those of Cantarow (1944) who observed that with the Malloy & Evelyn technique, when obstructive jaundice serum was mixed with haemolytic jaundice serum the observed proportion of direct bilirubin was greater than the theoretical. However, his haemolytic jaundice serum contained only 2·3 mg. bilirubin/100 ml. and of this only 13–22 % took part in the direct reaction. In our opinion, such measurements approach the limit of accuracy for the method and it is questionable whether the difference between theoretical and observed values for direct bilirubin are significant. Barron (1931) observed that the addition of bilirubin to normal sera caused the direct reaction to be negative, biphasic or prompt according to the amount of bilirubin added. Our earlier experiments have shown that in regurgitation jaundice, either delayed, biphasic or prompt reactions were obtained according to the concentration of bilirubin in the serum. Our experiments have also shown that even in haemolytic jaundice a weak direct reaction is always obtained and all the recent literature dealing with measurements of the alleged proportions of direct and indirect bilirubin show that at least 10 % of the total bilirubin takes part in the direct reaction in this condition. Thus, if the amount of bilirubin added is sufficiently large (over 15 mg./100 ml.) 1·5 mg./100 ml. or more, will take part in the direct reaction, and in the less diluted solution in

which the direct reaction is performed the reaction will appear by a qualitative test to be positive or even prompt. The observations of Barron (1931) are thus explicable.

SUMMARY

1. The rates of azobilirubin production from bilirubin-containing body fluids have been determined under various conditions.

2. In the presence of a phosphate buffer containing urea, all sera from patients with regurgitation jaundice, all but five of sixty-five sera from patients with retention jaundice and all but three of the twelve pleural fluids examined, formed azobilirubin rapidly on treatment with the diazo reagent.

3. In the presence of a phosphate buffer but not containing urea, the diazo reagent formed azobilirubin rapidly from all sera of patients with regurgitation jaundice, but much more slowly from most sera of patients with retention jaundice.

4. High concentrations of urea catalyze and increase azobilirubin production to a greater extent in retention jaundice than in regurgitation jaundice.

5. The difference between measurements of direct bilirubin by the Malloy & Evelyn method and by that of Rappaport & Eichhorn is due not only to the difference in pH at which the measurements are made, but also to the presence of urea in high concentration in the buffer used in the latter method.

6. In the presence of sodium citrate, citric acid, caffeine and urea, the diazo reagent formed azobilirubin rapidly from all sera from patients with regurgitation jaundice and about one-third of the sera from patients with retention jaundice, but more slowly from the remaining two-thirds. Haemorrhagic pleural fluids behaved similarly to the sera from patients with retention jaundice.

7. In retention jaundice, when azobilirubin production is plotted against time, a series of curves is obtained, some of which are identical with those observed in regurgitation jaundice.

8. These experiments suggest that the rapid formation of azobilirubin on the addition of diazo reagent to sera from patients with regurgitation jaundice is due to the presence of catalysts in the sera and not the existence in regurgitation jaundice of a form of bilirubin different from that in serum of patients with retention jaundice.

9. The behaviour of the diazo reagent towards bilirubin added to normal sera and to sera containing pathological constituents, or physiological constituents in excess, suggests that the catalytic substance or substances possibly in combination with the bilirubin may be specifically elaborated in regurgitation jaundice.

10. The terms direct and indirect bilirubin should be abandoned because there is no proof that two different forms exist.

11. The use of the term direct-indirect quotient is recommended since it is merely an index of the extent of the direct reaction.

Our thanks are due to all those clinicians who have given us such free access to their cases. Grants from the Central Research Fund of the University of London, and from the Medical Research Council enabled special necessary apparatus and chemicals to be purchased. Particular thanks are also due to Mr A. H. Dawson and Miss M. Sandiford for their able technical help and to Dr E. ff. Creed who read the manuscript and made valuable suggestions.

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The Thermal Destruction of Vitamin B_1

3. THE INFLUENCE OF COPPER ON THE RATE OF DESTRUCTION OF ANEURIN IN BUFFER SOLUTIONS AT 100°

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The first recorded statement concerning the influence of copper on the rate of destruction of aneurin is that of Booth (1943), who claims that aneurin is destroyed more rapidly in the presence of Cu than in its absence. Work in these laboratories on the behaviour of aneurin during the manufacture of yeast extract which contained significant amounts of Cu suggested that, perhaps, this metal was not such an accelerant of aneurin destruction as might be expected from Booth's statement. It was decided, therefore, to use the methods employed in earlier vitamin B_1 destruction studies (Farrer, 1945 *a*, *b*) for a more detailed investigation of the influence of Cu on the rate of destruction of aneurin.

Booth reported that aneurin in phosphate-phthalate buffer at pH 5·4 was destroyed more rapidly when 2 p.p.m. of Cu were added to the solution in the form of $CuSO_4$, but this is the only experiment recorded in his paper. The work herein describes the behaviour of aneurin in various buffer solutions at pH values between 3 and 7 in the presence of 2 and 20 p.p.m. of Cu, and, as in the earlier papers, the determination of the unimolecular reaction constant, *k*, in each case has permitted a quantitative comparison of the results. It has been found that the effect of Cu is variable.

EXPERIMENTAL

Reagents

Buffer salts. Because of the influence of very small amounts of heavy metals on aneurin destruction (q.v.) and the difficulty in general of obtaining satisfactory reagents for this type of work by recrystallization, the following solutions were made from A.R. reagents and distilled water and purified by the $CaCO_3$ adsorption method described by Stout & Arnon (1939): M-Na_2HPO_4, 0·5M-sodium potassium tartrate, 0·5M-glycine, potassium hydrogen phthalate-K_2HPO_4 (0·1M with respect to each salt). The purified solutions were filtered, and a measured volume of the filtrate was just acidified with the 6N-HCl (q.v.) and boiled for 15 min. to expel CO_2. When cool the solution was adjusted to the original volume with twice distilled water. The solutions were tested for heavy metals by means of the dithizone method and showed contamination of the order of considerably less than 0·1 p.p.m. in the concentrated solutions. This was considered to be satisfactory especially as the reagents were diluted at least tenfold before use in experiments upon aneurin destruction.

Citric acid. An attempt to purify sodium citrate in the same way was not entirely successful. A solution (0·5M) was prepared from some citric acid which had been thrice recrystallized. Buffer solutions with Na_2HPO_4 were tested for heavy metals by evaporating a large sample (70 ml.) and ashing before proceeding with the dithizone test. The result obtained was completely negative and consequently this citric acid (0·5M solution) was used with confidence.

6N-*Hydrochloric acid.* HCl (A.R.) was twice distilled from all-glass apparatus.

Twice distilled water. Doubly distilled water was prepared in all-glass apparatus with a 'strip action' still. This water was used in the preparation of all the solutions.

Copper solution. Copper sulphate (0·795 g. of A.R. $CuSO_4.5H_2O$) was dissolved in distilled water (250 ml.). When 5 ml. of this solution were diluted to 200 ml., the resulting Cu concentration was 20 p.p.m. Concentrations of 2 p.p.m. were obtained by appropriate dilution of the $CuSO_4$ stock solution.

Other metal solutions. Solutions of $FeNH_4(SO_4)_2.12H_2O$, $ZnSO_4.7H_2O$ and $NiSO_4(NH_4)_2SO_4.6H_2O$ were made up with distilled water so that 5 ml. contained 200 mg. of each metal (the Fe solution was slightly acidified with 6N-HCl to remove opalescence). Hence when 5 ml. were diluted to 200 ml. the resulting solution contained 1 p.p.m. of metal.

Aneurin stock solution. Pure aneurin hydrochloride (10 mg.) was dissolved in twice distilled water, acidified with 2 or 3 drops of 6N-HCl, and the solution diluted to 250 ml.

Method

The Jansen thiochrome method was used and the apparatus and procedure were the same as previously described (Farrer, 1941, 1945*a*).

Procedure

In the case of the phosphate buffer, 20 ml. of the purified, acidified solution and 4 ml. of the aneurin stock solution were pipetted into a 200 ml. volumetric flask. In appropriate cases 5 ml. of Cu solution of suitable concentration were also added. (In the confirmatory experiments 5 ml. of either Fe, Zn or Ni solutions were substituted for the Cu solution.) After dilution to about 170 ml. with twice distilled water, the pH was adjusted to the desired value by the addition of the 6N-HCl, and the solution was made up to volume. The concentration of the final solution was thus 0·1M with respect to phosphate. The final pH was checked; pH determinations were performed with the glass electrode.

The other buffer solutions were prepared in a similar manner. 40 ml. of mixed phosphate-phthalate solution were used to give a final concentration of 0·02M with respect to each salt, and 20 ml. of the Na_2HPO_4 formed the basis for the remaining three solutions which, by adding 8 ml. of tartrate, citrate or glycine solution in addition to the phosphate, were thus 0·1M with respect to phosphate and 0·02M with respect to the other buffer constituents when diluted to the final volume.

The prepared solutions were then boiled in a flask fitted to a reflux condenser by means of a ground-glass joint. At regular intervals portions (about 20 ml.) of the boiling solution were pipetted into 50 ml. conical flasks and cooled rapidly under the tap. Samples (5 ml.) were then taken for analysis by the thiochrome method. At the conclusion of the experiment the pH was again checked. Both glass and quinhydrone electrodes were used in this work.

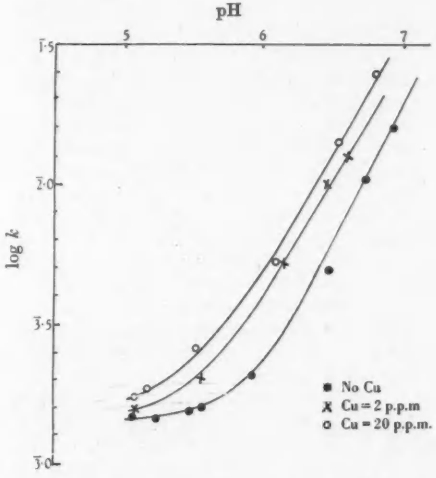

Fig. 1. pH/log k curves for aneurin destruction in phosphate solutions with and without the addition of Cu (k = velocity coefficient for the thermal destruction of aneurin).

RESULTS

As in the earlier work, the galvanometer readings are equivalent to $(a-x)$ in the monomolecular reaction equation:

$$k = \frac{2{\cdot}303}{t} \log_{10} \frac{a}{a-x}$$

and $\log_{10}(a-x)$ was plotted against time. From the resulting straight lines, k has been calculated for each experiment and graphs have been drawn of log k against pH (see Figs. 1–8).

In the phosphate (Fig. 1) and the phosphate-phthalate (Fig. 2) buffer solutions Cu at a concentration of 2 p.p.m. or 20 p.p.m. accelerates aneurin destruction at all acidities. It should be stated that the effective concentration of Cu in the solutions to which 20 p.p.m. of Cu were added was a good deal less than this figure as, on boiling, precipitation of $Cu_3(PO_4)_2$ was brought about by the large excess of phosphate ions.

In phosphate solutions to which tartrate, citrate or glycine were added both acceleration and deceleration of aneurin destruction have been encountered (Figs. 3, 4 and 5). With all three substances the same results were generally obtained with 20 p.p.m. of Cu as with 2 p.p.m. although, at the higher pH values for phosphate-glycine solutions the effect of 2 p.p.m. of Cu tends to disappear.

A complicating factor in the solutions used in this work is, however, the presence of comparatively large amounts of phosphate and a precipitate identified as $Cu_3(PO_4)_2$, was encountered in most of the tartrate solutions to which 20 p.p.m. of Cu were added.

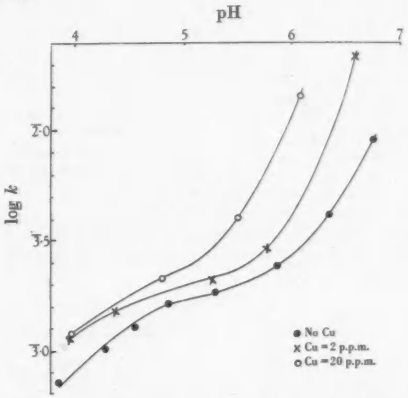

Fig. 2. pH/log k curves for aneurin destruction in phosphate-phthalate solutions with and without the addition of Cu.

Experiments were therefore carried out to investigate this precipitation. Solutions of acidity between pH 4 and 6 were made up to contain Na_2HPO_4, sodium potassium tartrate and $CuSO_4$ in the concentrations of 0·1, 0·02 and 0·005M respectively and were adjusted to the desired pH with HCl. The extinction (derived from Beer's law) which is indicative of complex formation (q.v.) was measured and the solutions were then boiled for 1 hr. No precipitate was obtained at pH 4·40 but a precipitate did appear in the solutions at pH 5·15, 5·50 and 5·85 and was identified as $Cu_3(PO_4)_2$ precipitated by the common ion effect of a large excess of phosphate ions as in the phosphate and phosphate-phthalate solutions described above. The extinction of the solution at pH 4·40 was unchanged, that of the solution at pH 5·15 was reduced by about two-thirds and those of the solutions of pH 5·50 and 5·58 were virtually nil.

There were no precipitates with the citrate phosphate buffer (Fig. 4) and aneurin destruction is retarded by Cu from pH 3–6·5. There is a hint of

acceleration above pH 6·5 but the rate of aneurin destruction generally is so rapid even slightly above pH 7 that the matter was not investigated further.

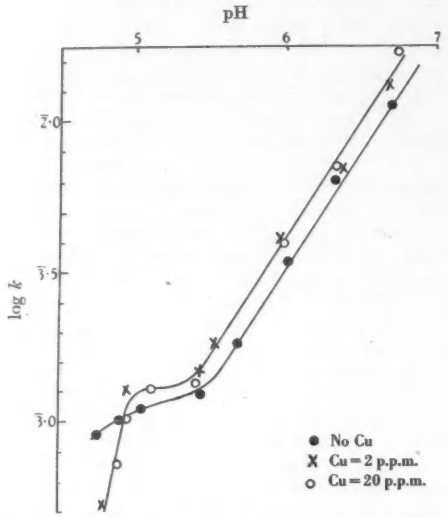

Fig. 3. pH/log k curves for aneurin destruction in phosphate-tartrate solutions with and without the addition of Cu.

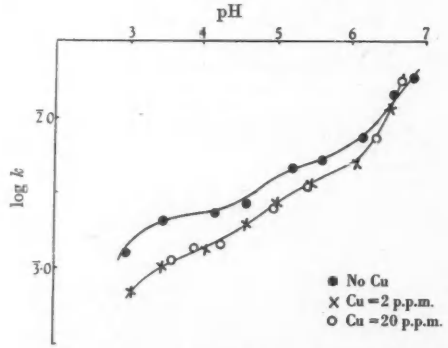

Fig. 4. pH/log k curves for aneurin destruction in phosphate-citrate solutions with and without the addition of Cu.

An attempt made to use unbuffered solutions for studying the effect of Cu before the part played by the Cu complexes was realized gave unsatisfactory results. The interchangeability of Na_2HPO_4 and K_2HPO_4 has been demonstrated from time to time and $CuCl_2$ gave exactly the same results as $CuSO_4$.

DISCUSSION

From the results, Cu can both accelerate and retard the destruction of aneurin, according to the conditions obtaining in the solution. While acceleration only has been observed in phosphate and phosphate-phthalate buffer solutions, both acceleration and deceleration occur in solutions containing tartrate, citrate or glycine. These compounds are among those with which Cu forms complexes. It is suggested that the retarding effect with Cu found in the solutions containing them is due to the presence of these complexes.

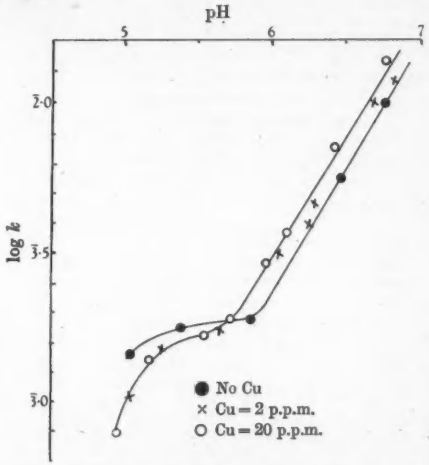

Fig. 5. pH/log k curves for aneurin destruction in phosphate-glycine solutions with and without the addition of Cu.

Wark (1923, 1924, 1927) studied the complexes formed by Cu with hydroxy-acids and describes acids with anions containing Cu. Speaking of Cu malate he states that, although the normal salt is said to preponderate in solution, there is always a sufficient concentration of hydrogen and cupri-malate ions to give the characteristic properties of this anion. By analogy, the same author postulated the existence of cupri-salicyclic, -glycollic and -lactic acids in equilibrium with the Cu salts in solutions of which the pH values are 3·5, 4·7 and 4·7 respectively. Bobtelsky & Jordan (1945) support this by reporting that Cu (and Ni) complexes with citric and tartaric acids behave as monobasic acids, so there is good reason for assuming the presence of cupri-citrate and cupri-tartrate anions in the solutions studied in this work. It is also likely that the large excess of citrate and tartrate over Cu in these solutions will favour the formation of the complex anion.

From the behaviour of boiling phosphate-tartrate-$CuSO_4$ solutions recorded above it would seem that at pH values between 5 and 6 the solubility product of $Cu_3(PO_4)_2$ is small compared with the dissociation constant of the complex and hence the tendency is for the latter not to be formed. Nevertheless, there would appear to be sufficient Cu left in solution to give the same results as are obtained with solutions containing 2 p.p.m. of Cu in which there is no precipitation. It is difficult to imagine free Cu^{++} in solutions containing such an excess of tartrate and when solutions 0·02 and 0·005M with respect to sodium potassium tartrate and $CuSO_4$ respectively were boiled at pH 4·54, 5·11 and 5·86 for 1·5 hr. there was no precipitation of the normal salt and no apparent change in extinction so that the cupri-tartrate seems to be relatively stable at 100° under conditions more nearly approaching those obtaining in the aneurin studies. The acceleration of aneurin destruction by Cu over most of the range studied and the deceleration below pH 5 are therefore likely to be due to the complex cupri-tartrate anion, the variation in behaviour being a pH effect conditioned by the phosphate which seems to determine the general shape of the pH/log k curves.

The case of the citrate-phosphate buffer (Fig. 4) is more straightforward. The absence of precipitation suggests that the cupri-citrate complex is more stable than the cupri-tartrate and it is submitted that the deceleration of aneurin destruction (see Fig. 4) may be ascribed to the presence of the copper-citrate complex anion.

Borsook & Thimann (1932) present evidence for the existence of several complexes of the amino-acids glycine and alanine with Cu in dilute solution at pH values from about 2 upwards. In the pH range studied in the present work, particularly since the glycine concentration greatly exceeds that of the Cu, it seems that two complexes are encountered: the so-called 'second acid complex', which, according to Borsook & Thimann, can exist at pH 3–6, and the 'neutral copper-glycine', which is said to predominate in the range pH 5–8. In the pH/log k curve for the phosphate-glycine solutions (Fig. 5) the similarity to the curve for phosphate solutions is clearly seen, but, with the addition of Cu, it is apparent that the reaction velocity is modified by the Cu complexes to show a deceleration of aneurin destruction below pH 5·7 and an acceleration above this pH. No precipitates were encountered in these solutions.

If the explanation for the variable effect of Cu on aneurin destruction is the occurrence of complex anions containing Cu, complex anions containing other metals might also be expected to modify the rate of aneurin destruction. Booth mentions that Fe, Sn, Zn and Al were without effect on aneurin destruction but he seems to have used phosphate-phthalate buffer solutions.

Reference to Fig. 6 shows that in phosphate solutions Fe, Zn and Ni have no effect on the rate of destruction of aneurin. However, in phosphate-citrate solutions these metals, for the most part, are

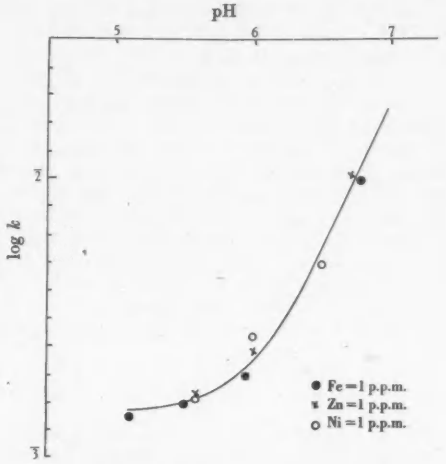

Fig. 6. pH/log k curve for aneurin destruction in metal-free phosphate solutions (with points omitted for convenience: see Fig. 1) on which are superimposed points for similar solutions containing Fe, Zn and Ni.

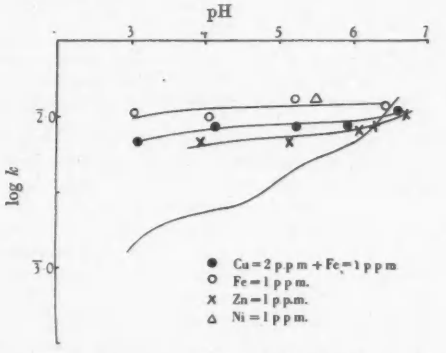

Fig. 7. pH/log k curve for aneurin destruction in phosphate-citrate solutions without added metals compared with similar curves obtained in the presence of Fe, Zn, Ni and a Cu-Fe mixture.

accelerants (Fig. 7) although the pH/log k curves are nearly horizontal. Bobtelsky & Simchen (1939) refer to an iron-citrate complex anion $(Fe_2Cit_3)^{=}$, which is formed in the presence of excess citrate and is said to be very stable, and reference to a zinc citrate complex has been made by Bobtelsky & Jordan (1945). Ni has not been studied as closely as Fe in

the present work but has shown acceleration of the same order as the latter at pH 5·5. Bobtelsky & Jordan (1945) found Ni complexes with citrate analogous to those with Cu. These results are thus an extension of the earlier work in that it seems that, with the exception of the special case of acceleration by Cu^{++}, it is the presence of various anions which cause the variations in the behaviour of aneurin.

This work affords an explanation of some results obtained when this investigation was first begun. Since there was no reason for suspecting that Fe or any heavy metals other than Cu, for that matter, catalyzed aneurin destruction, and, in fact, Booth had reported specifically that several had no effect, recrystallized reagents were used and tests for Cu only were carried out on the solutions by the addition of sodium diethyldithiocarbamate. With such solutions a pH/log k curve was obtained for aneurin destruction in phosphate-citrate solutions in the presence of 2 p.p.m. of Cu. In Fig. 8 it is compared

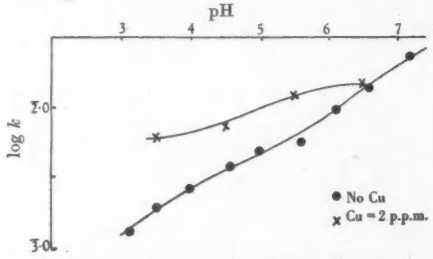

Fig. 8. pH/log k curves for aneurin destruction in phosphate-citrate solutions, with and without added Cu, made from recrystallized but insufficiently purified reagents.

with a similar curve without added Cu. The rate of aneurin destruction has been considerably accelerated. With the specially purified reagents described herein a series of experiments was carried out in which 2 p.p.m. of Cu and 1 p.p.m. of Fe were added to the aneurin containing phosphate-citrate solution (Fig. 7). The results obtained were close to the earlier curve for the solution containing Cu, i.e. aneurin destruction was accelerated but not to the same extent as with Fe alone. It seems therefore that the earlier solutions were contaminated with traces of Fe and that the behaviour of the aneurin is the result of a compromise between the two metal complexes present.

Further confirmation of the ideas put forward herein is found in the fact that Cu retarded the rate of aneurin destruction in solutions of yeast extract which contain amino-acids with which it is capable of forming complexes.

The method of preparing the buffer solutions used in this work inevitably leads to their contamination with Ca. However, the pH/log k curves for phosphate and phosphate-phthalate solutions purified by $CaCO_3$ adsorption are exactly the same as curves obtained with solutions of thrice recrystallized reagents which must be very low in Ca, used in the preliminary part of this work and in other work (Farrer, 1947). Moreover, when $CaCl_2$ (Ca = 20 p.p.m.) was added to the latter solutions, there was no change in the rate of destruction of the aneurin. However, Bobtelsky & Jordan (1945) report that Ca can form a complex with citrate although the equilibrium is usually in favour of the free ions and precipitation of calcium citrate. In solutions so dilute with respect to Ca it is hard to say what is the position and the results obtained with the specially purified phosphate-citrate solutions show small variations from the results obtained with the recrystallized reagents. These may possibly be due to a Ca effect but are more likely to be due to traces of heavy metals, which give more stable complexes, in the latter, and the presence of Ca has therefore been ignored in interpreting the results obtained.

From time to time abnormally fast rates of destruction have been encountered. Booth has already mentioned this point and there appears to be no obvious explanation of it. Similarly, in the absence of any indication of an oxidation of aneurin, there appears to be no simple explanation for the mechanism of the accelerating effect of Cu.

SUMMARY

1. Methods already described have been used to study the effect of two different concentrations of Cu (2 p.p.m. and 20 p.p.m.) on the rate of destruction of aneurin in the presence of five different buffer solutions at pH values from 3 to 6·8.

2. In phosphate and phosphate-phthalate solutions Cu accelerates the rate of destruction of aneurin at all pH values studied, greater acceleration being obtained with the greater concentration of Cu.

3. In phosphate solutions containing tartrate, citrate or glycine both acceleration and deceleration of aneurin destruction have been brought about by Cu and the variations in reaction velocity have been attributed to the presence in the solutions of complex anions containing Cu which act as positive or negative catalysts of aneurin destruction.

4. Confirmation of this explanation has been found in the fact that, while Fe, Zn and Ni are without effect on aneurin destruction in phosphate solutions, all three metals can modify the reaction velocity in phosphate solutions containing citrate with which all three can form complex anions.

The author wishes to thank the Directors of Kraft Walker Cheese Company Proprietary Limited for permission to publish this paper and gladly acknowledges the assistance of Messrs A. G. McKeon, S. L. Blencowe and, particularly, R. G. Miller in the large number of aneurin assays carried out.

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The Thermal Destruction of Vitamin B_1

4. THE INFLUENCE OF THE CONCENTRATION OF BUFFER SALTS ON THE RATE OF DESTRUCTION OF ANEURIN AT 100°

By K. T. H. FARRER, *Research Division, Kraft Walker Cheese Co. Proprietary Ltd., Melbourne, Australia*

(*Received* 22 *July* 1946)

In the course of the work on the influence of copper (Farrer, 1947), it was noticed that aneurin was destroyed more slowly in buffer solutions considerably more dilute than those used in earlier experiments (Farrer, 1941, 1945 *a*, *b*). The influence of the concentration of buffer salts on the thermal destruction of aneurin has therefore been investigated further, with the methods already developed. It has been found that the concentration of buffer salts can, under certain conditions, greatly influence the rate of destruction of aneurin but that the effect can vary in different buffer solutions.

EXPERIMENTAL

Reagents

The work reported herein was carried out before the effect of copper upon the thermal destruction of aneurin had been completely investigated (Farrer, 1947). Consequently reagents purified by recrystallization and not $CaCO_3$ adsorption were used. However, this work has been allowed to stand as the solutions were copper-free and reaction velocity determinations with 0·1 M-phosphate and 0·02 M-phosphate-phthalate solutions purified by $CaCO_3$ adsorption gave results exactly comparable with those obtained with the recrystallized reagents. Moreover, the results of Farrer (1947) show that other heavy metals (Fe, Zn and Ni) have no effect on aneurin destruction in the absence of complex-forming anions, and Booth (1943) could find no effect on aneurin destruction by adding Fe, Sn, Al or Zn to phosphate-phthalate solutions.

Phosphate buffer solutions. $Na_2HPO_4.12H_2O$ was thrice recrystallized from glass-distilled water. A 0·2 M solution was prepared with glass-distilled water and diluted as required for the various concentrations. The pH was adjusted with glass-distilled 6 N-HCl.

Phosphate-phthalate buffer solutions. Samples of potassium hydrogen phthalate and KH_2PO_4 were twice recrystallized from glass-distilled water and a stock solution 0·2 M with respect to each salt was made up. A slight flocculent precipitate, identified as an Al salt was always obtained no matter how carefully the salts were recrystallized (cf. Clark, 1928). Repeated determinations of k carried out in the presence of 5 p.p.m. of Al gave the same results as were obtained in its absence (as indicated by the alizarin spot test) which confirmed Booth's result. Accordingly, the precipitate was removed by filtration. The pH was adjusted to the desired value with KOH (copper-free).

Phosphate-citrate buffer solutions. A 0·1 M solution of the citric acid used by Farrer (1947) was prepared with glass-distilled water. The 0·2 M-$Na_2HPO_4.12H_2O$ solution described above was used for these solutions too.

The aneurin stock solution and the method of assay were as described by Farrer (1947).

Procedure. In the case of the phosphate-citrate buffer, 20 ml. of solution of the desired pH were prepared by pipetting the appropriate volumes of the citric acid and phosphate solutions into a 200 ml. volumetric flask; 4 ml. of aneurin solution were added, and the solution was made up to volume giving a tenfold dilution of the McIlvaine buffer used in the earlier work (Farrer, 1945*a*). The final pH was measured.

The preparation of the phosphate and phosphate-phthalate solutions and the subsequent procedure were the same as described by Farrer (1947).

RESULTS

The method of interpretation is the same as was used in the earlier work. For the phosphate (Fig. 1) and phosphate-phthalate (Fig. 2) solutions pH/log k curves are given and in Figs. 3 and 4, k has been plotted against the salt concentration for solutions of

constant pH. With the phosphate-citrate solutions no difference in reaction velocity at the greater dilution could be detected at pH 3, 3·5, 4, 4·5, 4·75, 5·04, 6·01 and 7·09. Hence it was not thought necessary to reproduce the pH/log k curve which has already been presented (Farrer, 1945*a*).

above pH 6 its rate of destruction appears independent of the concentration of the buffer salts within the limits studied. However, it is in the phosphate-phthalate buffer solutions that the concentration of the buffer salts is found to have the greatest effect. At all pH values between 3·9 and 7, the rate of

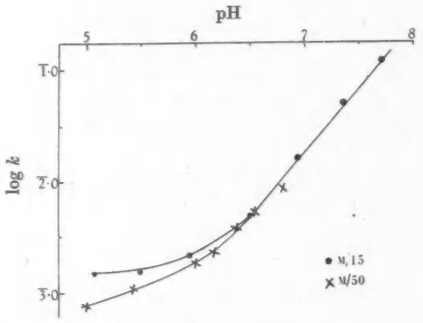

Fig. 1. pH/log k curves for aneurin destruction in phosphate solutions of two different concentrations (k = velocity coefficient for the thermal destruction of aneurin).

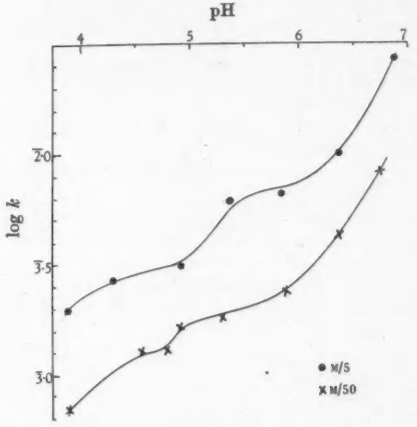

Fig. 2. pH/log k curves for the destruction of aneurin in phosphate-phthalate solutions of two different concentrations.

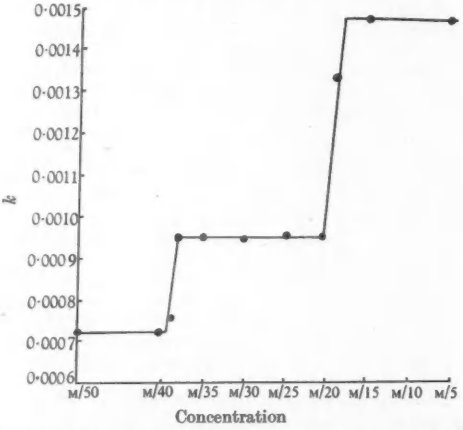

Fig. 3. Concentration/k curve for the destruction of aneurin in phosphate solutions at pH 5·04 (±0·04).

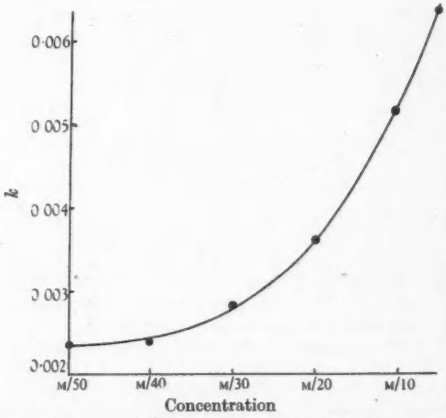

Fig. 4. Concentration/k curve for the destruction of aneurin in phosphate-phthalate solutions at pH 5·90 (±0·04).

It is evident from Figs. 1 and 2 that the concentration of the buffer salts affects profoundly the rate of destruction of aneurin. Thus, in direct contrast with the phosphate-citrate solutions, aneurin is destroyed progressively more slowly in the phosphate solutions below pH 6 as the pH falls, although

aneurin destruction is much less in the 0·02M solutions than in the 0·2M. In addition, the step in the pH/log k curve for the 0·2M solution has almost disappeared at the lower concentration.

The course of this variation in the rate of destruction of aneurin as the salt concentration was in-

creased was studied; pH 5 for phosphate solutions, and pH 5·9 for phosphate-phthalate solutions, were selected as convenient acidities at which to work. With both solutions, the velocity coefficient, k increases as the concentration of the solution is increased. In the phosphate solutions, the rate of aneurin destruction rises, at critical concentrations, through a series of three levels as the concentration is increased from 0·02 to 0·2 M (Fig. 3). On the other hand, in the case of the phosphate-phthalate buffer, k increases continually with the concentration over the range studied (Fig. 4).

DISCUSSION

It is apparent that the behaviour of aneurin in the presence of phosphate is varied by the citrate and phthalate ions. Citrate appears to suppress the effect of concentration altogether, and phthalate has altered the relationship between concentration and reaction velocity. This serves still further to emphasize the influence of the various buffer ions on the thermal destruction of aneurin.

The concentration effect has also been found in solutions of yeast extract in which a sudden rise in reaction velocity from one level to another at an almost critical concentration was observed. The concentration/k curve was very similar to that found with the phosphate buffer solutions, although it consisted of only one 'step' and the values of k were of a different order from those obtained with the last-mentioned buffer at comparable concentrations.

SUMMARY

1. The effect of the concentration of the buffer salts on the rate of destruction of aneurin was studied in three different buffer solutions between the concentrations 0·2–0·02 M and the pH values 3·9–7·0.

2. In phosphate buffer solutions the velocity coefficient, k, is dependent on the concentration of the buffer salts below pH 6. This effect appears to be eliminated by citrate in the phosphate-citric acid buffer solutions and magnified by phthalate in phosphate-phthalate buffer solutions.

3. In phosphate buffer solutions at pH 5, the velocity coefficient rises sharply at critical concentrations of about M/39 and M/19 through three levels when the concentration is increased from 0·02 to 0·2 M. However, in phosphate-phthalate buffer solutions at pH 5·9, the relation between the velocity coefficient and concentration changes continuously over the same concentration range.

The author thanks the Directors of the Kraft Walker Cheese Co. Pty. Ltd., for permission to publish this paper.

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The Effect of Age and of the Supply of Phosphorus on the Assimilation of Calcium by the Rat*

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(*Received* 16 *September* 1946)

Young growing rats retain Ca with almost 100% efficiency from diets suboptimal in this element but adequate in P (Ellis & Mitchell, 1933; Fairbanks & Mitchell, 1936; Henry & Kon, 1939). The last named authors found that under most favourable conditions the daily loss of Ca by a young rat could be as low as 0·3 mg. but that the daily excretion of P could not be brought below 1·3 mg.

* Read in part before the Biochemical Society (Henry & Kon, 1945).

In those experiments the diets contained suboptimal but appreciable quantities of Ca and one aim of the present study was to find whether with much lower intakes this element would be held with equal tenacity, and to determine the amount of P needed to promote its most efficient utilization.

For this purpose the P content of a diet originally very low in Ca and P (diet 191) was raised by addition of $Na_2HPO_4 . 12H_2O$ to a level sufficient to cover the minimum unavoidable loss of this element

and to deposit as bone all the Ca present, but insufficient to cover fully the requirements of the soft tissues (diet 192). By doubling this amount of total P, a diet (diet 199) was obtained, in which P was in no way a limiting factor, while by halving it, diet 198 was produced containing only about enough P to balance the unavoidable loss. The retention of Ca and P from these diets was compared with that from the basal diet and also from a diet (diet 193) containing a higher level of Ca and P derived from milk and from sodium phosphate, which had been shown previously to promote an optimal retention of Ca (Henry & Kon, 1939).

About 99 % of the body Ca is in the bones and, as the animal normally matures, a gradual decrease in the retention of this element can reasonably be expected to accompany the progressive mineralization of the skeleton. Bloom (1930), for example, found that rats 33 days old retained 93 % of Ca from a diet containing 0·45 % Ca and 0·34 % P, but that rats 63 days old retained only 58 %. Nicolaysen (1943) reported similar observations. In the experiments of Lantz & Smith (1934) the retention of Ca from the milk-wheat diet (diet B) of Sherman & Macleod (1925) fell from 77 % at 1·5 months to 39 % when the rats were 3 months old.

Fairbanks & Mitchell (1936), Rottensten (1938), Nicolaysen (1943) and other workers have shown that for growing animals the efficiency of utilization of Ca depends primarily on the degree of saturation of the body reserves. Hence with adequate diets it will be closely related to the age of the animal during the period of growth. It seems likely, however, that, quite apart from the Ca content of the tissues, the stage of development of the organism exerts a definite effect. A further aim, therefore, of this inquiry has been to study the gradual changes in assimilation of Ca by the rat as the animal progresses from adolescence to senility.

The metabolic tests used in the first part of this study were therefore repeated with the same rats at intervals for 2 years. During the periods of rest the animals received a stock diet supplying liberal amounts of Ca.

At the completion of the main experiments further tests were done with special diets to investigate peculiarities of metabolism of Ca by old rats.

EXPERIMENTAL

Plan

The experiment was run in two series. The performance of rats receiving diets 191, 192 and 193 was studied in one, that of rats receiving diets 198, 192 and 199 in the other. Diet 192 was common to both and served as a connecting link. Further diets were tested with the 2-year old rats.

Diets

The basal diet low in Ca and P (diet 191) was that used by us previously (Henry & Kon, 1937, 1939), slightly modified. The composition of this diet and of the other diets derived from it is given in Table 1. To minimize scattering all diets were offered mixed with distilled water. The drinking water also was distilled. The following vitamins were given daily in solution separately from the diet: aneurin, 10 μg.; riboflavin, 40 μg.; pyridoxin, 10 μg.; Ca pantothenate, 300 μg.; inositol, 1 mg.; nicotinic acid, 1 mg.; choline hydrochloride, 3 mg.; *p*-aminobenzoic acid, 3 mg., and cod-liver oil, 2 drops.

Table 1. *Composition of the 'synthetic' experimental diets*

(Amounts of components in diet are given as parts.)

Component	Diet 191	Diet 198	Diet 192	Diet 199	Diet 193	Diet 233	Diet 232
Egg-white, commercial, heat-coagulated	13·5	13·5	13·5	13·5	12·0	14·0	21·0
Sugar, granulated ground	9·0	9·0	9·0	9·0	8·0	10·0	10·0
Maize starch	64·8	64·8	64·8	64·8	57·7	53·28	48·68
Margarine fat	10·0	10·0	10·0	10·0	9·9	18·0	13·0
Ca- and P-free salts (Henry & Kon, 1937)	2·7	2·7	2·7	2·7	2·4	3·0	3·0
Spray-dried skim milk	—	—	—	—	10·0	—	—
$Na_2HPO_4.12H_2O$	—	0·12	0·48	1·11	1·38	1·39	2·94
$CaHPO_4.2H_2O$	—	—	—	—	—	0·33	1·38

The stock diet, given during the rest intervals was as described by Folley, Ikin, Kon & Watson (1938). With it tap water was given to drink. Records of consumption of this dry diet, fresh milk and liver have allowed us to calculate the mean composition of the food eaten by stock colony rats. This is given in Table 2. The Ca content would be augmented by that of the tap water which in this locality amounts to 0·0425 g. Ca/l.

Table 2. *Composition of diet* 228

	(%)
Stock diet	70·7
Dried full-cream milk	26·5*
Dried liver	2·8†

* Equivalent to 208 ml. liquid milk.
† Equivalent to 10 g. fresh liver.

The following additional diets were given to the old rats: (1) the stock colony diet. To simplify feeding, sampling and analysis, fresh milk was replaced by whole dried milk and the liver was steamed, minced, dried, ground and mixed

with the basal diet and milk. This was diet 228. (2) Diet 232, a 'synthetic' type diet, similar in fat, protein, Ca and P content to diet 228. (3) A dried mixed human diet (Macrae, Henry & Kon, 1943). Its Ca and P content was very similar to that of diet 193. (4) Diet 233, a 'synthetic' diet similar to the mixed human diet.

With all these diets the rats were given distilled water to drink; the 'synthetic' diets were supplemented with vitamins as already described. Their compositions are given in Tables 1 and 2 and their analyses in Table 3.

Table 3. *Analyses of the experimental diets**

Diet no.	Ca (%)	P (%)	Ca : P ratio	Moisture (%)
191	0·0114	0·0180	0·63	10·34
198	0·0114	0·0290	0·39	10·07
192	0·0117	0·0592	0·20	10·45
199	0·0113	0·1131	0·10	10·58
193	0·1290	0·2291	0·56	10·84
Mixed human	0·1194	0·2207	0·54	8·74
233	0·1204	0·2190	0·55	7·72
228	0·4610	0·5926	0·78	9·95
232	0·4607	0·5684	0·81	9·30

* A fresh batch of each diet was made and analyzed for each experiment. There were small variations from batch to batch. The figures quoted here are the means for all batches.

Animals

In each series of tests four groups of three, and one group of six, littermate male rats of our own breeding (Folley *et al.* 1938) were used. At the start they were 24–27 days old in the first series and 26–29 days in the second. The initial weights were respectively 37–43 and 43–51 g.

Technique

Littermates were chosen by lot to receive the different diets which were offered in individual metabolic cages. The excreta voided during the first 7 days of any test were discarded and the collection periods lasted 24 days in the first series of experiments and 21 in the second. Metabolic tests were done at the beginning and again when the rats were about 6 months, 1 year and 2 years old. In the first test, six rats were given each diet but in the remaining tests only four animals were used per diet as not more than twelve large metabolic cages of the Hopkins type (Ackroyd & Hopkins, 1916) suitable for adult rats were available at a time. The spare rats were kept as possible substitutes. Several rats died later in the experiment and it proved impossible to follow the original intention of using throughout the same groups of littermates. The error thus introduced was overshadowed by the large differences between the effects of various treatments. During the periods of rest when the rats received the stock diet they were housed four together in ordinary cages. The metabolic cages and the methods of collecting and analyzing the excreta have been described in an earlier publication (Henry & Kon, 1937).

RESULTS

The mean results of the experiment are given in Table 4. It may be convenient to review them under separate headings.

A. *The metabolism of* Ca *and* P *in the period of vigorous growth*

Calcium. With diet 191 very low in Ca and P the young rats lost Ca from their bodies at a rate of 3·4 mg. daily, most of it in the urine, the faecal excretion amounting to only 0·1 mg. which is as little as under conditions for optimal retention, e.g. with diet 193 (cf. Henry & Kon, 1939). The urinary loss of Ca diminished with increasing concentration of P in the diet, until for diet 199, which supplied nearly seven times as much P as diet 191, it was only 0·3 mg. daily, rather less than with diet 193 and little more than the minimum observed by us under best possible conditions (Henry & Kon, 1939). It is noteworthy that the faecal excretion was not affected by these changes in intake of P. Diet 192 which contained half as much P as diet 199 apparently supplied just enough of the element to prevent loss of Ca from the body. In fact with the batch of diet used in the first series of experiments there was a small negative balance, while in the second series the diet proved slightly richer in P and the balance became positive.

Phosphorus. The daily loss of phosphorus with the diet deficient in Ca and P (diet 191) was as low as that observed by us previously under conditions presumably conducive to optimal retention (Henry & Kon, 1939). Again the urine contained only 0·1 mg. P daily while ten times this amount, 1·2 mg., was found in the faeces. So little P was present in the diet that, despite the smallness of the excretion, the rats were in negative balance, losing about 0·2 mg. P daily from their bodies. With all other diets the balance was positive, the amount retained daily rising steadily from 1·0 mg. from diet 198, containing twice as much P as diet 191, to over 11 mg. daily from diet 193 with a P content over ten times higher.

B. *Changes in the metabolism of* Ca *and* P *with progressing age of the rat*

Calcium. As would be expected, the retention of Ca became less favourable as the rats grew older. Where, from the start, the balance was negative, as with the low Ca and P diet, with diet 198 and, in one series of experiments, with diet 192, the magnitude of the loss increased steadily. For diet 191, for example, the daily loss of 2·7 mg. at 4 weeks rose to nearly 5 mg. at 1 year and to nearly 6 mg. at 2 years.

With two other diets low in Ca but with added P, diet 192 used in the second series and diet 199, the balance was at first positive. It will, in fact, be recalled that from the latter diet the young rats retained Ca with optimal efficiency. In both instances the balance later became negative and

Table 4. *Mean values for the* Ca *and* P *metabolism of rats, receiving, at different ages, diets of varying* Ca *and* P *content*

(All values, unless otherwise stated, given on a daily basis.)

Rats used: Age at start of test (weeks)	No.	Length of test (days)	Wt. of rat at start of test (g.)	Gain (g.)	Diet intake* (g.)	Ca intake (mg.)	Ca excreted (mg.): Urine*	Ca excreted (mg.): Faeces*	Ca retained (mg.)	Ca retained (%)*	P intake (mg.)	P excreted (mg.): Urine*	P excreted (mg.): Faeces*	P retained (mg.)	P retained (%)*
Diet 191†, first series															
4	5	24	64·8	0·71	6·57±0·16	0·78	3·31±0·26	0·11±0·01	− 2·66	−344·8±28·6	1·15	0·12±0·004	1·21±0·10	− 0·18	− 16·8±10·8
26	4	24	279·7	0·43	17·21±0·43	2·08	1·78±0·24	2·75±0·12	− 2·45	−118·5±10·5	3·51	0·56±0·069	3·73±0·12	− 0·78	− 22·7± 6·5
56	4	24	370·5	0·28	17·95±0·30	1·92	3·17±0·56	3·67±0·51	− 4·92	−256·2±10·7	3·11	0·41±0·064	4·22±0·30	− 1·52	− 48·9±10·1
93	3	24	318·3	−0·76	13·48±1·66	1·48	2·66±0·41	4·75±0·35	− 5·93	−409·3±46·2	2·27	1·46±0·670	4·66±0·27	− 3·86	−185·4±61·5
Diet 198†, second series															
4	6	21	85·7	1·25	9·56±0·39	1·15	3·11±0·24	0·26±0·02	− 2·22	−193·1±15·7	3·07	0·31±0·16	1·80±0·10	+ 0·96	+ 29·9± 7·5
26	3	21	276·7	0·87	14·90±0·52	1·60	1·56±0·50	2·08±0·45	− 2·04	−128·8±26·1	4·23	0·59±0·26	3·40±0·28	+ 0·24	+ 4·6±15·9
55	4	21	383·5	−0·21	17·92±0·73	1·95	1·25±0·08	4·90±0·16	− 4·20	−217·3±18·3	5·08	2·45±0·16	5·15±0·17	− 2·52	− 51·0±10·2
93	4	21	384·8	−0·92	17·21±1·84	2·07	1·19±0·05	4·65±0·20	− 3·77	−191·9±30·5	4·71	2·10±0·67	5·38±0·26	− 2·77	− 65·7±26·4
Diet 192†, first series															
4	5	24	69·8	1·52	7·72±0·20	0·99	1·10±0·12	0·09±0·01	− 0·20	− 19·8±10·0	4·44	0·53±0·13	1·46±0·08	+ 2·45	+ 54·9± 3·7
26	4	24	284·5	0·60	17·16±1·09	2·06	1·07±0·10	3·61±0·32	− 2·62	−121·0±21·7	10·54	7·61±0·57	4·13±0·10	− 1·20	− 11·7± 2·0
56	4	24	397·0	0·80	19·25±0·41	2·33	1·21±0·05	5·01±0·51	− 3·89	−168·5±28·8	11·51	8·42±0·28	4·87±0·29	− 1·78	− 15·7± 5·8
93	4	24	410·0	−0·04	17·13±0·59	1·90	1·20±0·09	5·33±0·14	− 4·63	−244·2±13·7	9·73	7·13±0·31	5·10±0·12	− 2·50	− 25·8± 2·2
Diet 192†, second series															
4	6	21	85·5	1·87	9·73±0·29	1·16	0·53±0·13	0·16±0·01	+ 0·47	+ 41·1±11·2	5·93	0·69±0·13	1·70±0·06	+ 3·54	+ 59·5± 2·7
26	3	21	306·0	1·05	16·66±0·14	1·81	0·87±0·06	2·41±0·39	− 1·47	− 80·8±23·6	9·80	5·80±0·16	3·82±0·03	+ 0·18	+ 1·6± 1·4
55	4	21	394·2	0·02	16·97±0·94	1·81	1·20±0·06	4·92±0·45	− 4·31	−244·7±43·9	9·89	7·57±0·35	5·23±0·17	− 2·91	− 31·3±12·0
93	4	21	422·0	−0·19	18·54±0·51	2·22	1·19±0·21	5·37±0·22	− 4·34	−196·0±18·8	10·98	7·67±0·52	5·71±0·22	− 2·40	− 22·0± 2·6
Diet 199†, second series															
4	6	21	88·0	1·90	10·11±0·34	1·19	0·29±0·02	0·15±0·01	+ 0·75	+ 63·2± 0·8	11·83	5·80±0·18	1·94±0·12	+ 4·09	+ 34·5± 1·1
26	3	21	305·3	0·86	15·24±1·39	1·62	0·77±0·02	2·20±0·31	− 1·35	− 83·5± 9·1	16·54	13·32±1·33	3·37±0·17	− 0·15	− 1·1± 0·9
55	4	21	436·2	−0·19	18·65±1·27	2·05	1·20±0·08	5·19±0·24	− 4·34	−219·2±26·1	20·94	17·46±1·84	5·03±0·23	− 1·55	− 7·9± 2·4
93	4	21	394·3	−1·32	17·80±1·66	2·12	1·12±0·14	5·09±0·20	− 4·09	−201·2±30·5	20·39	16·72±1·11	5·18±0·19	− 1·51	− 9·1± 6·7
Diet 193†, first series															
4	5	24	73·6	3·28	10·24±0·49	12·99	0·35±0·04	0·11±0·02	+12·53	+ 96·4± 0·6	23·18	10·12±0·44	1·77±0·12	+11·29	+ 48·6± 0·50
26	4	24	351·3	0·67	18·96±0·58	24·29	2·86±0·10	16·03±0·31	+ 5·40	+ 22·1± 1·8	44·97	32·88±1·12	9·00±0·33	+ 3·09	+ 6·8± 0·83
56	4	24	455·2	0·70	20·79±1·17	26·91	3·30±0·21	20·90±0·86	+ 2·71	+ 9·9± 1·7	47·54	32·75±1·77	11·78±0·57	+ 3·01	+ 6·2± 0·69
93	3	24	409·7	−0·04	18·82±0·95	24·78	4·80±1·54	21·63±0·45	− 1·65	− 6·7± 1·4	42·17	31·09±2·74	13·91±0·77	− 2·83	− 5·1± 2·84
Diet 228†, first series															
102	8	14	364·1	−0·44	16·05±0·54	74·27	3·86±0·58	69·64±1·94	+ 0·77	+ 0·96±0·6	96·67	40·13±1·53	53·21±1·38	+ 3·33	+ 3·5± 1·7
Diet 228†, second series															
108	5	14	363·6	−0·63	14·60±1·11	67·09	3·67±0·97	61·14±4·23	+ 2·28	+ 3·4± 1·3	85·12	37·25±4·11	46·69±2·73	+ 1·18	+ 1·4± 1·0
Diet 232†, second series															
108	5	14	343·4	−0·57	13·56±1·33	62·45	2·42±0·32	58·99±6·43	+ 1·04	+ 2·1± 2·0	77·04	39·46±4·46	36·82±4·52	+ 0·76	+ 1·7± 1·9
Mixed human diet†, second series															
113	4	14	348·2	−1·73	11·67±1·30	13·93	1·81±0·56	14·26±1·74	− 2·14	− 14·9± 5·0	25·76	18·23±2·72	10·53±0·54	− 3·00	− 12·1± 3·6
115	4	14	324·0	−0·74	12·63±1·76	15·07	2·72±0·58	14·85±1·72	− 2·50	− 19·4± 8·2	27·86	18·37±3·18	8·46±1·46	+ 1·03	+ 3·1± 3·0
Diet 233†, second series															
113	3	14	353·7	−1·29	12·52±0·73	15·08	1·85±0·37	15·36±0·46	− 2·13	− 14·4± 3·9	27·43	20·56±0·73	9·36±0·41	− 2·49	− 9·6± 6·3
115	3	14	335·7	−1·93	12·09±0·37	14·55	2·74±0·44	13·07±0·73	− 1·26	− 6·9± 8·5	26·47	19·16±1·57	8·34±0·42	− 1·03	− 3·7± 5·5

deteriorated as the rats grew older. For example, the daily retention from diet 199 of 0·8 mg. Ca by 4 weeks old rats changed to a loss of 1·4 mg. at 6 months and of over 4 mg. at 1 and 2 years of age. It is of interest that, while the retention of Ca by young rats from the low Ca diet was markedly affected by the P content of the diet, rats over a year old lost about 4 mg. daily whether the diet contained 0·03 or 0·11 % P, though those receiving the diet with only 0·02 % P lost 5–6 mg.

The most striking effect of age on the assimilation of Ca was undoubtedly that observed with diet 193, suboptimal in Ca, but much more adequate in this respect than any of the other diets, and with ample phosphorus. From the almost quantitative retention, amounting to 96 % at the stage of rapid growth, the assimilation decreased steadily, as would be expected, until at 1 year old the rats were almost in Ca equilibrium, retaining only some 3 mg. daily, or 10 % of the intake. We had expected that they would thereafter remain in Ca balance. In fact, however, a year later the rats were losing Ca from their bodies at the rate of nearly 2 mg. a day while receiving the same diet from which they had retained it so efficiently in their early youth.

Further tests were done with these rats to find out whether the negative balance was an unavoidable effect of senility or if it could be prevented by the use of a diet richer in Ca and P. The latter proved true with the modified stock diet (diet 228) which maintained the rats in Ca equilibrium.

As this diet is composed of natural ingredients, the question arose whether a 'synthetic' diet (diet 232) would do equally well. Parallel tests were therefore done with littermates in the second series of experiments. These showed no difference between the two diets (228 and 232), thus indicating that the maintenance of Ca equilibrium in old age is primarily a question of the supply of Ca and P. This view was strengthened when it was found in tests which followed that a reversion to a diet of lower Ca content led to a loss of Ca from the body whether the diet was 'natural' or 'synthetic'. The 'natural' diet was the dried human diet described by Macrae *et al.* (1943) and the 'synthetic' ration (diet 233) was similar to it in fat, protein, Ca and P content.

In discussing with us the results of our experiments, Dr D. P. Cuthbertson suggested that the negative Ca balance observed with old rats receiving diet 193 might have been due to their taking longer than when young to reach equilibrium after a change in diet, and that it was possible that the 7 days' preliminary period after the switch-over from the stock diet given during the rest period to diet 193 was, for them, too short. If this were so, a prolongation of the experimental period should restore Ca balance. To test this point the comparison of the 'natural' and the 'synthetic' rations was continued for another 2 weeks directly on completion of the tests described above. Table 4 shows that the loss of Ca persisted unchanged with both diets. It seems therefore that the inefficient retention of Ca by old rats is not due to delayed acclimatization but is inherently connected with senescence. In all cases the deterioration was largely due to less efficient uptake from the gut. The old rats consistently lost weight during all the metabolic tests; whatever the effect of this loss on the retention of minerals it applied equally to all results.

Phosphorus. The retention of P followed generally the Ca trends. The percentage retention from all diets decreased as the rats grew older with the completion of growth of skeleton and active tissue. The increased loss was mostly in the urine especially with the diets containing the larger amounts of P derived from sodium acid phosphate.

DISCUSSION

A. *The metabolism of* Ca *and* P *in the period of vigorous growth*

Calcium. It may be stressing the obvious to cite our experiments as a demonstration of the intimate metabolic interrelation of Ca and P. We do this without apology as this truism has often been glaring enough to be overlooked. The influence of the one mineral on the utilization of the other is indeed striking as shown so clearly now by the effect of an increasing supply of P on the retention of Ca by the young growing rat. In fact, while with a diet containing only 0·01 % Ca and 0·02 % P there was a heavy drain of Ca from the body, an increase of the P content to 0·1 % led to a positive balance of Ca despite the minuteness of the supply. We had previously shown in agreement with other workers (Henry & Kon, 1939; Ellis & Mitchell, 1933) that with suboptimal but substantial supplies of Ca the retention may be so good that only unavoidable traces are lost from the body. The present findings show that this is equally true when the diet is much poorer in Ca, provided other conditions are conducive to maximum retention. For the period of vigorous growth in the rat we cannot therefore confirm the view expressed by Smith & Smith (1934) that 'there is a constant metabolic demand—a "wear and tear" requirement—for calcium operative even when there exists an absolute lack in the diet'. This ability to retain all but traces of Ca may only last a short time. Our rats were already at 6 months excreting so much more Ca that they were in negative balance with the low Ca diet just mentioned. The rats of Smith & Smith (1934) were about 4 months old and it may well be that their findings would have been similar to ours had their rats been younger.

Phosphorus. For the growing animal P is in a sense even more essential than Ca as the former is needed in more nearly equal proportions for the formation of bone and of extra-skeletal tissues, while 99 % of the Ca is concentrated in the bones. We have previously shown (Henry & Kon, 1937, 1939) that when the supply of both minerals is inadequate the soft tissues take precedence over the bones in respect of P. At 4 weeks of age the rats receiving the various diets were all growing though at rates increasing with the P intake. Those receiving diet 191 were actually losing P from their bodies, the others were retaining roughly from 1 to 4 mg. P/g. gain in weight. For the calculation of the amount of P available for the formation of the soft tissues the amounts retained in, or liberated from, the bones must be taken into account. This we have done by assuming that 99 % of Ca is in the bones, that the Ca : P ratio of bone is 2·3 : 1 (Brooke, Smith & Smith, 1934; Kramer & Shear, 1928), and that the bone minerals are liberated in that ratio in negative Ca balance, and deposited in positive. Values so obtained combined with the P balance give the P available for extra-skeletal use. Table 5 shows the

Table 5. *Calculated amounts of* P *available for deposition in the soft tissues of young rats during the period of vigorous growth* (4 *weeks*) *and after the cessation of vigorous growth* (26 *weeks*)

	4-weeks old rats		26-weeks old rats
Diet no.	mg. P available/g. gain in wt.	% of normal requirement	mg. P available/g. gain in wt.
191	1·37	52·1	0·70
198	1·54	58·6	1·29
192 (1st series)	1·67	63·8	−0·12
192 (2nd series)	1·87	68·2	0·77
199	1·98	75·7	0·50
193	1·80	68·7	1·15

results of such calculations. The relative constancy of the amount of P deposited in the soft tissues/g. gain in weight is remarkable in view of the wide divergency of the exogenous supply. A similar calculation applied to the values of Sherman & Quinn (1926) for 'normal' rats of the same age gives a retention in the soft tissues of 2·62 mg. P/g. gain in weight. Rats in the present experiment satisfied, therefore, their extra-skeletal requirements to an extent varying from 52·1 % for diet 191 to 75·7 % for diet 199. More direct earlier measurements based on carcass analysis yielded (Henry & Kon, 1939) a figure of 85 % for a diet similar in composition to diet 193, but by the present method of calculation, the only one possible in the absence of carcass analysis, the figure is reduced to 77 %. The indirect method yields, therefore, lower figures.

The large excess of P necessary to promote the optimal retention of Ca is worthy of note. For diet 199, with which these optimal conditions were apparently reached, the P : Ca ratio was 10 : 1. This marked increase in P with the Ca remaining reasonably constant did not affect the faecal excretion of the latter but brought down the urinary loss from 3·31 (diet 191) to 0·29 mg./day (diet 199). If bones had precedence over the soft tissues in the competition for P, optimal retention of Ca could presumably be achieved with an amount of P sufficient to retain as bone the Ca otherwise excreted in the urine with diet 191. The additional quantity required for this purpose can be calculated as

$$\frac{3{\cdot}31 - 0{\cdot}29}{2{\cdot}3} = 1{\cdot}3 \text{ mg. P/day.}$$

As diet 191 supplied 1·15 mg. P/day, the total needed would amount to 2·45 mg. P/day. In fact 11·8 mg. proved necessary, and the intermediate amounts of 3·1 mg. supplied by diet 198 and 4·5–6·0 mg. supplied by diet 192 were insufficient to prevent a marked loss of Ca in the urine. It seems evident that only a fraction of the P was available under these conditions for binding Ca in the bone, or that, in other words the demands of the soft tissues were 'setting the pace'.

The calculations of P available for soft tissues were based on the assumption that in negative Ca balance the Ca is derived from the bone minerals as a whole with a corresponding release of the P. Brooke *et al.* (1934) have shown, however, that, under certain conditions, the Ca is preferentially withdrawn from the bone carbonate. Their diet contained rather more P than our diet 199 with which the Ca balance was positive and the question of P derived from bones did not arise. Whether their findings apply to the diets and conditions used by us is an open question. It might well be argued on teleological grounds that the marked loss of Ca with diets deficient in both Ca and P is part of a mechanism intended to supply P for growth of soft tissues by the dissolution of bone. That this loss of Ca is not unavoidable is shown by its becoming negligible in the presence of sufficient P. A withdrawal of Ca from the carbonate fraction of bone only would seem purposeless under such conditions.

B. *Changes in the metabolism of* Ca *and* P *with progressing age of the rat*

Calcium. In our experiments, the increasing inefficiency of Ca retention is the striking feature of advancing age. It will be recalled that during periods of rest between metabolic tests all the rats received the fully adequate stock diet. The Ca stores of the adult rats were therefore probably saturated at the beginning of each experiment and the degree of

saturation was in all probability the same for rats 1 year old as for those 2 years of age, yet the former managed to maintain Ca equilibrium with diet 193 whereas the latter did not. Increasing katabolism of the skeleton in senescence is the most likely explanation of this difference, and indeed indications are not lacking from animal experiments or from observations on man of the loss of bone salts in old age (McCay, Crowell & Maynard, 1935; Meulengracht, 1938; Todd, 1942; Stare, 1943). In the vigorous anabolic phase of growth, deposition of bone elements is possible even with low concentrations of these elements in the diet. As the skeleton matures, the flow in the direction of the skeleton becomes more sluggish and finally stops. After a period of equilibrium, the tide turns with advancing age but the ebb can be stopped, as our experiments show, by raising the level, and thus increasing the potential, of the bone minerals in the diet. If adequacy of supply can be measured by rate of retention or, later, by maintenance of equilibrium, two peaks of Ca need will be found, one in youth and the other in senility. There is much evidence to show that a good supply of Ca is advantageous for the growing animal (Toepfer & Sherman, 1936; Sherman & Campbell, 1937; Lanford, Campbell & Sherman, 1941; Campbell & Sherman, 1945). It is less certain whether maintenance of Ca equilibrium in advanced age is equally desirable. In senility Ca tends to deposit in tissues other than the bone, and it may be that this is a direct result of the bone atrophy, as in the mobilization of bone salts in intoxication with vitamin D. If that were true the damming of bone katabolism by increased supply of Ca and P in the diet might well prove beneficial. It might on the other hand itself contribute directly to the aberrant deposition of Ca.

Phosphorus. By 6 months the growth of the active soft tissues in the rat is nearly completed and further gain in weight is largely due to deposition of fat. It is therefore not surprising that a calculation of the P available for soft tissues/g. gain in weight (p. 174) should yield figures much more variable than those obtained at the stage of vigorous growth (Table 5). Calculations for rats 1 and 2 years old are meaningless since by then growth had entirely stopped or the rats were actually losing weight.

On the whole the retention of P followed with age the pattern of the retention of Ca. Again, for each diet the balance became increasingly less satisfactory as the animals grew older. An increase in the supply of Ca and P for old rats re-established a positive balance.

SUMMARY

1. The Ca retention by rats from diets containing 0·011 % Ca and 0·018–0·113 % P has been studied at intervals in two series of experiments from the time the rats were 1 month old until they attained the age of 2 years. In one series a simultaneous comparison was made with a diet containing 0·129 % Ca and 0·229 % P (diet 193). Between metabolic tests the animals were maintained on a stock diet containing adequate Ca and P.

2. Young actively growing rats were in negative Ca balance on the diet containing 0·011 % Ca and 0·018 % P. With increasing P content of the diet Ca retention improved and at 0·059 % P the animals were just in balance, while at 0·113 % P they retained 63 % of the ingested Ca. The daily excretion of 0·44 mg. Ca was almost identical with the figure of 0·46 mg. obtained with diet 193, considered from previous experience to induce optimal Ca retention.

3. Poorer Ca absorption, as shown by increased faecal Ca, was observed with all diets as the age of the rats increased from 1 month to 2 years. This was most striking with diet 193 where a positive Ca balance of 96·4 % at 1 month became negative to the extent of 6·7 % at 2 years of age.

4. Two-year old rats could be brought into Ca balance by increasing the dietary Ca and P to 0·461 % and 0·593 % respectively. It was immaterial whether the diet was composed of 'natural' or of 'synthetic' foodstuffs.

5. The retention of P was roughly parallel to that of Ca.

6. The evidence suggests that in young, actively growing rats the soft tissues take precedence over bone in the competition for P.

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Studies on the more Soluble Complexes of Soil Organic Matter

1. A METHOD OF FRACTIONATION

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This paper is intended as the first of a series dealing with the so-called 'fulvic acid' fraction of soil organic matter. Fulvic acid (after Oden, 1919) is that part of soil organic matter which is peptized by alkali but remains in solution on precipitation of the 'humic acids' with acid. It is proposed in this paper to set forth the method of fractionation adopted and in subsequent papers to deal in more detail with the character of the isolated complexes.

The fulvic acid fraction has not up to now received, in comparison with the humic precipitate, the attention it warrants. As it contains the more soluble soil organic constituents, it should be the fraction most intimately associated with plant growth, and thus be of the utmost importance. Moreover, from recent work by Tiurin (1944) it appears to be the fraction of soil organic matter which effects the mobilization of the sesquioxides, during podzolization.

Little work has been done on the actual isolation of the components of this fraction since Schreiner & Shorey (1909, 1910, 1911) isolated several simple degradation products (amino-acids, purine bases, sugars) in small yield. Since that early period, however, the methods of isolation of organic constituents from natural sources have been greatly improved and augmented.

Since the fulvic extract consists of water soluble components it was thought that it might be fractionated by a method using a chromatographic or general adsorptive technique. After tests under varying conditions with activated alumina, charcoal, various grades of fuller's earth and other clay adsorbents, charcoal was selected as the most suitable. The charcoal used was of the quality described as 'animal, purified' (Hopkin & Williams, Ltd).

It was found that if the fulvic extract was adjusted to pH 2·5–3·0 and then run through a layer of charcoal of suitable dimensions, over 90 % of the organic matter was adsorbed by the charcoal. This organic matter could be subsequently extracted with various solvents and thus separated into distinct fractions. The details of a method were determined and applied to the fulvic extracts of four soils.

EXPERIMENTAL

Materials

The soils used were as diverse as possible and consisted of samples of

(1) Heather raw humus,
(2) Gardener's 'turf' soil,
(3) *Sphagnum*-cottongrass peat,
(4) Pine raw humus.

Preparation of the fulvic extracts

A weight of the original oven-dried (at 65°) soil containing 100 g. of organic matter was stirred with 10 l. of 0·5 N-sodium hydroxide for 48 hr. It was then allowed to settle and the supernatant dark liquor siphoned off. This suspension was extremely difficult to filter by the usual methods. It was found, however, that when the solution was run through a wide cylinder packed with cotton wool to remove the finely suspended material it could be readily filtered under pressure through a sintered glass funnel (pore size 5–10 μ.). Filtration of 10 l. by this method took on the average only 24 hr. To the residue a further 10 l. 0·5 N-sodium hydroxide was added and the stirring, settling, and siphoning repeated. The residue was then washed by

adding and siphoning off 10 l. of distilled water. The combined filtrates and washings were acidified with hydrochloric acid and, after the 'humic' precipitate had settled, the acid solution was siphoned off. A sufficient excess of acid was added to retain the sesquioxides in solution. The humic precipitate was redissolved and reprecipitated as before, and the supernatant liquid added to the acid solution. In general, the total volume of the acid solution with washings from the humic precipitate was about 30 l. The pH of this solution was adjusted to 2·5–3·0.

With the exception of the 'turf' soil, the pigments present in the extracts could themselves be used as indicators for the adjustment of the pH, and this was confirmed by quinhydrone electrode determinations. The solutions were straw-yellow at a lower pH and wine-red at a higher pH, passing through an orange point in the pH range 2·5–3·0. With the mineral soil, however, the aluminium and ferric hydroxides present apparently affected the colour changes. At pH 2·5–3·0 the hydrogen ion concentration was sufficient to prevent the precipitation of these hydroxides. The solution at this and lower pH values was a deep red. At pH values near neutral, however, the hydroxides were precipitated along with coloured organic matter, leaving a straw-yellow solution.

The solution at pH 2·5–3·0 was filtered through a very fine paper to remove any sediment present.

Fractionation of the fulvic extract

The fulvic extracts from the four soils (30 l. at pH 2·5–3·0) were filtered in a Buchner funnel with suction applied through pads of animal charcoal, which had previously been thoroughly washed with 0·1 N-hydrochloric acid. The pads were about 2 in. in depth and 6 in. in diameter. All the colour and most of the organic matter was adsorbed on the charcoal. After the 30 l. of extract had passed through, the pad was washed with 500 ml. of 0·1 N-hydrochloric acid and the washings added to the filtrate.

Fraction A was composed of the colourless filtrate and washings.

The charcoal was then eluted successively with the following solutions:

Fraction B. Acetone containing 10% water was first run through the pad. The first 500 ml. of filtrate generally contained most of the colour extracted by this solvent. The extraction was continued until the solvent came through colourless. Generally about 4·5 l. of the solvent were required.

Fraction C. Distilled water was then sucked through. The extract was a clear colourless liquid, which gave a flocculent white precipitate on addition of acetone. The extraction was continued till the extract no longer gave a precipitate on addition of excess acetone. Generally about 1 l. of distilled water was sufficient.

Fraction D. The organic matter remaining adsorbed on the charcoal was then completely eluted with 0·5 N-sodium hydroxide. A deep wine-red eluate was obtained. In general 4 l. were sufficient to elute all the colour.

The fulvic extract was thus separated into four distinct fractions two of which (A and C) were colourless and two (B and D) coloured. One deduction that can immediately be drawn from these results is that there are at least two pigments present in the fulvic extract of markedly different properties.

Individual fractions

Fraction A. This fraction was not fractionated further for the following reasons. With all the materials examined only a very small part of the organic matter was not adsorbed on the charcoal. From a consideration of properties it was obvious that nearly all of the many simple organic substances (amino-acids, purine bases, sugars, etc.) isolated by Schreiner, Shorey and co-workers (1909–13) would occur in this fraction. The filtrate, unlike the original extract, no longer gave a precipitate with ethanol, phosphotungstic acid, or lead acetate, showing that all the organic complexes had been adsorbed. Nitrogen was determined on samples of the filtrate, and by comparison with the nitrogen present before adsorption it was found that 70–80% of the fulvic nitrogen had been adsorbed.

One litre portions of the colourless filtrates were carefully neutralized with sodium hydroxide. With the mineral soil there was a considerable precipitation of aluminium hydroxide on neutralization showing that the soluble aluminium salts had not been adsorbed. This alumina precipitate, unlike the precipitate obtained on neutralizing the original extract, contained no organic matter. The neutralized and, for the soil, filtered, solutions were taken to dryness under reduced pressure and extracted with ethanol. The residues left after the ethanol extraction contained no organic matter. The ethanol was then removed under reduced pressure and the residues extracted again with ethanol which after evaporation left in every case only a very small amount of a light brown syrup. This syrup was rich in nitrogen and gave a strong α-naphthol test for sugars. It reduced Fehling's solution and gave the phloroglucinol and orcinol tests for pentoses.

The study of this fraction was not continued further. It should, however, be pointed out that by this method the simple degradation products are easily obtained separately from the complex and colouring matters, and the fraction could, therefore, be worked up without the tedious use of metallic precipitants and clarifiers required for Schreiner & Shorey's (1910) method.

Fraction B. The first 500 ml. portion of the eluate from each of the four soils, was a deep wine-red colour. Generally, a small amount of a white flocculent precipitate, which proved identical with the material eluted with distilled water, formed in this first portion. The reason for this is presumably that at the start of the elution the acetone was first rapidly adsorbed by the charcoal, leaving the water it contained to elute this fraction but, since the charcoal soon became saturated with acetone, the acetone ran through and precipitated in the filtrate jar the water-eluted contaminant.

This material was filtered off to be added to the water-eluted fraction C. The total acetone eluate was transferred to a distillation apparatus and the acetone distilled off on a water-bath at 50–60°. The water was then removed under reduced pressure at the same temperature and the residue extracted with absolute ethanol.

All the colour and almost all the organic matter was soluble in ethanol; only a very slight colourless residue of a carbohydrate nature, with a very high ash (NaCl) content was left. This gave positive α-naphthol, phloroglucinol, and orcinol tests. The peat was the only material which gave a sufficient amount of this residue for a carbon estimation and the result, 38% C, agreed with the carbohydrate properties of the material which was nitrogen free.

The ethanolic solution of the bulk of the fraction was evaporated down under reduced pressure and excess ether added. The dark red amorphous precipitate was filtered, washed with ether, then redissolved in ethanol and reprecipitated with ether several times for purification. It was finally dried *in vacuo* and ground to a red powder.

	Yield (g./100 g. original organic matter)
Heather raw humus	3·39
'Turf' soil	2·13
Peat	2·51
Pine humus	4·35

The yields quoted are minimal, i.e. the amounts actually isolated after purification.

These powders were readily soluble in water, ethanol and acetone. On standing in the presence of air, the dry powders and their aqueous solutions slowly darkened in colour till finally, with the powders, black tars were obtained. The fresh powders all gave positive Molisch, phloroglucinol and orcinol tests showing the presence of sugars, and in particular pentoses. Their aqueous solutions also gave dark red to purple colours with ferric chloride solution indicating the presence of phenolic compounds; on hydrolysis with dilute acid a dark-coloured precipitate formed and the remaining solution strongly reduced Fehling's solution.

It is obvious from these properties that the substances are phenolic glycosides. They had the following carbon contents:

	Heather raw humus	'Turf' soil	Peat	Pine humus
Carbon (%)	43·71	45·24	47·31	46·35
Ash (%)	2·51	3·06	2·62	2·34

The isolation of these compounds in the yields shown from four widely diverse types of soil organic matter demonstrates that a quantitatively important glycoside fraction is widely distributed in soil organic matter. Shorey (1913) isolated a glycoside in unstated yield from a New York soil.

The instability of these glycosides in air is interesting since it suggests that they may be intermediate products in the formation of humic acids. Both the carbohydrate and phenolic constituents are being investigated.

Fraction C. To the distilled water eluate which had been run through the charcoal, were added 3 vol. of acetone. A voluminous flocculent white precipitate was formed which was allowed to settle. The precipitate was washed with pure acetone by decanting, and then filtered in a Buchner funnel. To it was added the small precipitate obtained in fraction B.

The total precipitate was purified by redissolving in water, filtering, and reprecipitating with acetone. It was again washed with acetone by decanting, filtered, and finally washed with ether. It was then transferred to a Petri dish and dried *in vacuo* to a clear transparent gel membrane having somewhat the appearance of gelatin. It was brittle in thick sheets but flexible in thin sheets.

	Yield (g./100 g. original organic matter)
Heather raw humus	1·64
'Turf' soil	1·32
Peat	1·60
Pine humus	1·94

The physical appearance and properties of this substance tended to suggest that it was pure, and, moreover, identical from all four sources. This identity was confirmed since the material from all four sources on heating started to redden at 194° and decomposed to a black friable mass at 200°.

Depending on the method of drying the substance could be obtained in clear pliable sheets, as an easily powdered white mass, or as a hard brittle translucent gum. After drying it was but slowly dissolved by water, softening to a sticky gel before going into solution. It was readily soluble in alkali and was acid to litmus. It gave a titration equivalent of about 1500 when titrated with 0·02 N-sodium hydroxide. Its high molecular colloidal structure was confirmed by its inability to dialyze through parchment. After hydrolysis with acid the hydrolysate strongly reduced Fehling's solution. The usual sugar tests were applied. It gave a strong positive α-naphthol test confirming its carbohydrate nature. Both the Seliwanoff and mucic acid tests were negative showing that neither ketoses, nor galactose nor galacturonic acid, were present. It gave, however, a positive saccharic acid test showing that glucose and/or glucuronic acid units were present in its molecule. It also gave positive phloroglucinol and orcinol tests indicating the presence of uronic acids and/or pentoses. The solution obtained by

hydrolysis for 4 hr. with 2 % (w/v) hydrochloric acid gave a positive naphthoresorcinol test showing that glucuronic acid was a component. The carbohydrate gave a reddish purple colour with iodine solution. Obviously the material was a polysaccharide, containing glucose and glucuronic groups and perhaps also pentose groups.

This first isolation of a polyuronic acid from soils confirms the many indications of the presence of uronic units obtained by the study of the evolution of carbon dioxide from soils on heating with acids (Shorey & Martin, 1930; Norman & Bartholomew, 1943). This carbohydrate, which can be obtained pure and in relatively large yield from a wide variety of soils, should be of considerable importance in the study of soil organic matter. A vigorous chemical study of its structure by the methods of the carbohydrate chemist is obviously warranted.

Fraction D. This fraction contains all the substances present in the original fulvic extract that have not been eluted by the previous solvents.

The anode, cathode and central liquids and the precipitate gave a negative biuret test, indicating that no proteins or other polypeptides were present. Hobson & Page (1932) also failed to obtain a positive biuret test with the total fulvic extract and assumed that this was due to interference by the colour of the fulvic acid. Most of the solutions in the present study were only lightly coloured or colourless, but despite this, no proteins were observed.

In dealing with the bulk of fraction D the 5 l. of alkali solution were first subjected to ordinary dialysis in a bell jar, with parchment clamped over the wide end, suspended in a tank of running distilled water. The dialysis was allowed to continue till colour just started to come through the membrane after about 1 week. The pH values of the solutions were then about 8–9. These solutions could then be electrodialyzed to completion quickly, each 750 ml. lot requiring only 24 hr.

The precipitate formed at the anode membrane was filtered off in each case and its properties further studied. These precipitates would appear to be the main constituents in fraction D. They were dried *in vacuo* and ground to a brown powder.

Table 1. *Electrodialysis of soil fulvic fraction D*

Time (hr.)	Anode cell: Volume (ml.)	pH	Colour of solution	Centre compartment: Colour of solution	pH
0	1st 700	—	Colourless	Wine red	—
1	2nd 700	12·5	,,	,,	—
2	3rd 700	—	,,	,,	—
3	4th 700	12·0	,,	,,	—
4	5th 700	—	,,	,,	—
20	6th 700	11·2	Faint yellow	,,	—
27	7th 700	—	Deep yellow	Lighter red	—
45	8th 700	8·4	Red	Colourless solution	—
65	9th 700	—	Faint yellow	with ppt. on anode	—
90	10th 700	6·0	Colourless	membrane	6·27

The problem in isolating this fraction is to remove the alkali. Accordingly, electrodialysis of the fraction was studied.

A 750 ml. portion of the deep wine-red sodium hydroxide solution from the 'turf' soil fraction D was electrodialyzed in a three-cell dialyzer against parchment with running distilled water in the cathode cell. The anode liquor was changed frequently, the temperature being allowed to rise to a maximum of 60° and the current to 2 amp. The changes observed are shown in Table 1.

After 90 hr. the central compartment was washed out with distilled water and the suspension filtered. The precipitate in the centre cell was obviously a mixture. It deposited at the anode membrane as a colourless film streaked with a red resin-like material. The filtrate from the centre chamber contained little organic matter.

The coloured anode solutions (6–9, Table 1) were collected, but although quite richly coloured they were found on evaporation to contain little organic matter. Indeed, most of the organic matter was included in the precipitate at the anode membrane.

	Yield (g./100 g. original organic matter)
Heather raw humus	1·68
'Turf' soil	2·60
Peat	0·38
Pine humus	1·56

	Analysis: C (%)	H (%)	N (%)	Ash (%)
Heather raw humus	48·44	5·49	4·17	2·16
'Turf' soil	47·25	5·61	5·87	4·10
Pine humus	—	—	3·50	1·47

Although relatively rich in nitrogen, solutions of the substances did not give any positive protein tests. They gave, however, positive phloroglucinol and orcinol tests for pentoses and contained, moreover, considerable amounts of phosphorus. They were acidic, being readily soluble in alkali, and were precipitated from solution by ethanol, lead acetate,

and by neutralization in the presence of aluminium salts.

This fraction appears to have properties similar to fractions isolated by Shorey (1913), Bottomley (1919), and Wrenshall & Dyer (1941).

The fractionation of fulvic nitrogen

The separation previously discussed is represented diagrammatically in Table 2. Nitrogen was determined in each fraction and the distribution of the fulvic nitrogen between these fractions was calculated. It is readily seen that the greater part of the nitrogen complexes is concentrated in fraction D.

Table 2. *The distribution of the total fulvic nitrogen between the individual fractions*

Fulvic extract 100

Not adsorbed — Charcoal adsorbed — Eluted

Filtrate fraction A Colourless	Acetone fraction B Coloured	Water fraction C Colourless	Alkali fraction D Coloured
S 21·3	*S* 7·9	*S* 3·4	*S* 52·4
Pi 31·7	*Pi* 12·2	*Pi* 7·3	*Pi* 40·9
H 24·8			*H* 45·3
P 30·4			*P* 37·8

S = percentage soil fulvic nitrogen in fraction.
Pi = percentage pine humus fulvic nitrogen in fraction.
H = percentage heather humus fulvic nitrogen in fraction.
P = percentage peat fulvic nitrogen in fraction.

The application of the methods of fulvic fractionation to the hydrolyzable fraction of the humic precipitate

It was observed during the purification and fractionation of the humic precipitate, not reported here, that as much as 50 % of it could be hydrolyzed with dilute acid. It is generally considered that this fraction consists mainly of hemicelluloses, possibly from bacterial gums (Winogradsky, 1929) but it was thought that a proportion of the hydrolyzable material might consist of the fulvic constituents already studied. All these constituents are acidic polymers and all are strongly adsorbed from acidic solutions. It was thought extremely likely that the humic acid, with its very large surface area, might adsorb on precipitation large amounts of the fulvic constituents which would only be very slowly removed by reprecipitation. Accordingly the following experiment was performed.

Humic precipitate (200 g.), which had been obtained from the soil, and purified by redissolving and reprecipitating twice, was refluxed for 1 hr. with 8 l. of distilled water at 100°. The suspension was filtered and the residue washed, dried and weighed. The weight was 162 g., leaving 38 g., or 19 % of the original, for the soluble fraction. The first filtrate was subjected to the fulvic fractionation.

The residue was suspended in 8 l. of distilled water and neutralized with dilute ammonium hydroxide. The solution was then refluxed for 2 hr., acidified and filtered. The residue was again washed, dried, and weighed. It weighed 128 g., and 34 g., or 17 % of the original, had gone into solution; it still contained 24·6 % of material hydrolyzable with 2 % (w/v) HCl.

The second filtrate was also subjected to the fulvic fractionation. The fractions obtained from the humic precipitate had all the properties, already described, of the fulvic fractions. The yields are shown in Table 3.

Table 3. *Yield of fulvic fractions from soil humic precipitate*

	First extract		Second extract	
Fraction	Yield (g.)	Humic precipitate (%)	Yield (g.)	Humic precipitate (%)
B	15·64	7·82	8·08	4·04
C	1·92	0·96	Trace	—
D	5·20	2·60	12·57	6·28

From these results it is seen that a considerable part (in this case 21·7 %) of the humic acid precipitate consists of constituents normally found in the fulvic extract. These contaminants of the humic acid appear to be removed only to a small extent by reprecipitation, more so by boiling water and completely by hydrolysis with dilute acid.

The fulvic fractionation of the water-soluble fraction of soil organic matter

The chief criticism which has been levelled against workers isolating organic constituents from soils is that the products obtained may not be actual *in situ* constituents of the soil but artefacts produced by the extractants used. It may be argued that the fractions isolated from the fulvic extracts are formed by the alkali used as extractant. To refute this possibility it is necessary to find out whether the same constituents can be obtained by the use of an extractant unlikely to produce any changes in the products.

It was thought that they might be obtained simply by extracting soils with water, although naturally in much smaller yields. Accordingly, a weight of the 'turf' soil equivalent to 100 g. of organic matter was refluxed for 1 hr. with 4 l. of distilled water, and then filtered and washed with a further 1 l. of distilled water. The extract had a pH of 4·38. Portions of the extract were taken for determination of nitrogen and organic matter and the remainder subjected to the usual adsorption on charcoal after adjusting the pH to 3·0. The eluate was colourless. Its nitrogen and organic content were measured. The charcoal was then eluted with acetone, water and alkali as before and the yields obtained are shown in Table 4.

These extracts were much lighter coloured than those from the fulvic extracts but the products obtained had properties identical with those of the fulvic constituents previously discussed.

Table 4. *Fractionation of water-soluble fraction of soil organic matter*

Water-soluble organic matter (g./100 g. organic matter)		Water-soluble nitrogen (mg./100 g. organic matter)	
Total water extract	5·98	Total nitrogen	210
Fraction A	3·15		
Fraction B	0·38		
Fraction C	1·06		
Fraction D	1·14		

Nitrogen adsorbed (complex nitrogen) 37·6%.
Organic matter adsorbed (complex substances) 47·3%.

As might be expected, a much higher proportion of the simpler substances (fraction A) was observed here. These results show that the fulvic constituents are present in the soil as such. Fraction C especially is easily extracted by water.

SUMMARY

1. A considerable advance has been made in the study of the fulvic extract of soil organic matter. A new method has been introduced whereby the fulvic extract is separated by a selective adsorption technique into four distinct fractions.

2. These fractions have been obtained from the fulvic extracts from four widely diverse types of soil.

3. A preliminary examination of the chemical properties of the fractions has been made.

4. A pure polysaccharide containing uronic acid groups has been isolated from the four soils. The presence of a glycosidic fraction in the four soils has also been shown.

5. The fulvic nitrogen has been shown to be in part contained in nitrogenous acidic complexes and in part as simple degradation products.

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A Polarographic Study of Acid-insoluble Fractions from Iodo-proteins

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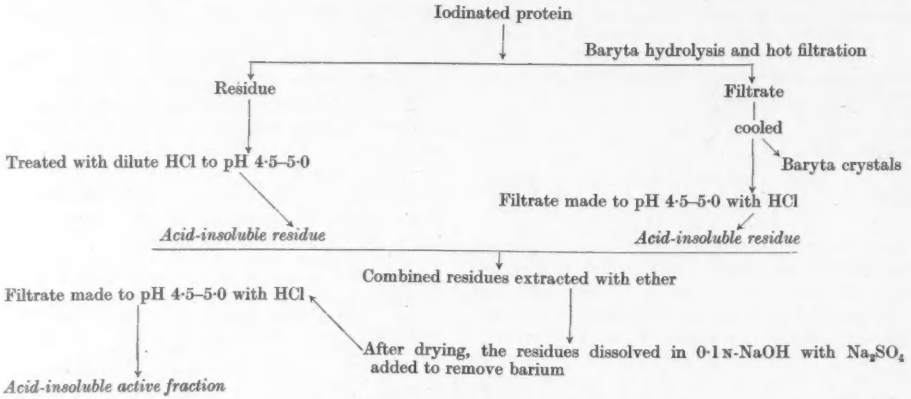
In the study of iodinated proteins, attempts to correlate the thyroxine iodine and the biological activity of these thyro-active substances have met with varied success. Deanesly & Parkes (1945) express the view that most of the acid-insoluble iodine as obtained by the accepted method (British Pharmacopoeia, 1932) is not true thyroxine iodine and that more evidence is required before the actual relationship between biological activity and thyroxine content can be measured.

More recently, Reineke & Turner (1945) claim to have obtained close correlation between biological activity and the thyroxine content of iodinated casein. The method adopted was to hydrolyze the iodinated protein in a baryta solution and to extract the hydrolysate with *n*-butanol. The thyroxine content was then calculated from iodine estimations done on the purified *n*-butanol extracts.

The present paper describes some preliminary work on the application of the polarographic wave of thyroxine to certain acid-insoluble fractions obtained from the baryta hydrolysis of iodinated proteins.

METHODS

Samples of iodinated 'Ardein'* (ground nut protein) and iodinated casein were hydrolyzed by baryta. A partially purified acid-insoluble active fraction was obtained by subsequent treatment according to the method of Ludwig & von Mutzenbecher (1939) as indicated in the following diagram.


Iodinated protein
Baryta hydrolysis and hot filtration
Residue
Filtrate
cooled
Baryta crystals
Treated with dilute HCl to pH 4·5–5·0
Filtrate made to pH 4·5–5·0 with HCl
Acid-insoluble residue
Acid-insoluble residue
Combined residues extracted with ether
Filtrate made to pH 4·5–5·0 with HCl
After drying, the residues dissolved in 0·1 N-NaOH with Na_2SO_4 added to remove barium
Acid-insoluble active fraction

The Cambridge Polarograph, the technique and the capillary characteristics used were similar to those described in the polarographic determination of thyroxine (Simpson & Traill, 1946). Half-wave potentials were there recorded against a saturated calomel electrode, but for the present analytical method the reference electrode used was the normal quiescent mercury pool anode.

RESULTS

Determination of apparent thyroxine in active fractions

When a small amount of an acid-insoluble active fraction, obtained from an iodo-Ardein, was dissolved in a mixture of 0·5 N-Na_2CO_3 and ethanol (3:2), a composite polarographic wave between $-1{\cdot}1$ and $-1{\cdot}9$ V. was obtained. Addition of tetramethylammonium bromide to the above test solution yielded a more clear-cut reproducible wave similar to that produced by pure thyroxine. When small quantities of pure thyroxine were added to the test solution, the wave height increased by proportionate amounts. In the following, it is assumed that thyroxine is the only substance in the acid-insoluble fraction which produces this characteristic wave structure. A calibration curve was made relating wave height and concentration of added thyroxine in a solution of the active fraction.

(*a*) *Acid-insoluble fraction from the baryta hydrolysis of iodinated Ardein* (PB 13). Active fraction (PB 13C) (0·52 g.) was dissolved in 52 ml. of a mixture of 0·5 N-Na_2CO_3 and ethanol (3 : 2), i.e. 1 ml. of solution contained 0·01 g. of PB 13C. A solution was prepared of 0·06 g. of thyroxine sodium B.P. in 20 ml. of the same ethanolic carbonate solution (1 ml. of this solution contained 0·003 g. of thyroxine sodium). Polarographic test solutions were prepared as in Table 1.

It is seen from Table 1 that each 10 ml. portion of test solution contains the same quantity of base electrolytes. Polarograms were recorded at a galvanometer sensitivity of 1/300, without removal of oxygen, and are reproduced in Fig. 1 *a*, *b*.

Table 1. *Polarographic test solutions*

No. of test solution	Solution PB13C (ml.)	Pure thyroxine sodium solution (ml.)	0·5 N-Na_2CO_3/ ethanol solution (3 : 2) (ml.)	Tetramethylammonium bromide (g.)
1	2	—	8	0·2
2	2	1	7	0·2
3	2	2	6	0·2
4	2	4	4	0·2
5	2	6	2	0·2
6	2	6·75	1·25	0·2
7	3	—	7	0·2
8	4	—	6	0·2

The relationship between the polarographic wave height and thyroxine sodium content is given in Table 2.

Table 2. *Relation between polarographic wave height and thyroxine content*

No. of test solution	Thyroxine sodium added (g.)	Polarographic wave height	Wave height difference
1	0·000	11·0	0·0
2	0·003	13·0	2·0
3	0·006	15·0	4·0
4	0·012	19·25	8·25
5	0·018	23·0	12·0
6	0·0203	24·5	13·5
7	—	15·75	4·75
8	—	20·25	9·25

* The word 'Ardein' is a registered trademark.

The figures in the last column of Table 2 were obtained by subtracting the wave height for test solution no. 1 from the values for the remaining solutions. The calibration curve relating thyroxine concentration with wave height difference is shown in Fig. 2 (*a*). From this curve, the quantity of thyroxine equivalent to the additional 1 ml. and 2 ml. of PB 13C solution in test solutions nos. 7 and 8 respectively can be determined. Thus (*a*) 1 ml. of PB 13C solution is equivalent to 0·00705 g. of thyroxine sodium, and (*b*) 2 ml. PB 13C solution are equivalent to 0·0139 g. of thyroxine sodium. The percentage of apparent thyroxine sodium in this active fraction is therefore (*a*) 70·5; (*b*) 69·5; average 70·0.

The percentage thyroxine sodium in this acid-insoluble fraction calculated from the iodine content assuming all the iodine present to be thyroxine iodine, was found to be 68·6. The iodine analysis was carried out according to the British Pharmacopoeia (1936 Addendum) method for total iodine determination (Table 3) on thyroxine sodium.

(*b*) *Acid-insoluble fraction from the baryta hydrolysis of iodinated casein* (PB 25). Active fraction (PB 25C), 0·2 g. was dissolved in 35 ml. of a mixture of 0·5 N-Na_2CO_3 and ethanol (3 : 2) (1 ml. of solution contained 0·00571 g. of PB 25C). A solution of thyroxine sodium in the same ethanolic carbonate solution containing 0·002 g./ml. was prepared. Polarographic test solutions were prepared in a similar manner to that used above in Table 1, omitting no. 6 test solution.

Polarograms were recorded without removal of oxygen and at a galvanometer sensitivity of 1/150. These are reproduced in Fig. 3 *a*, *b*. From the calibration curve, Fig. 2 (*b*), relating polarographic wave height and concentration of added thyroxine sodium in this case, the thyroxine quantities equivalent to the extra 1 ml. and 2 ml. of PB 25C solution were found to be 0·0046 and 0·00935 g. respectively.

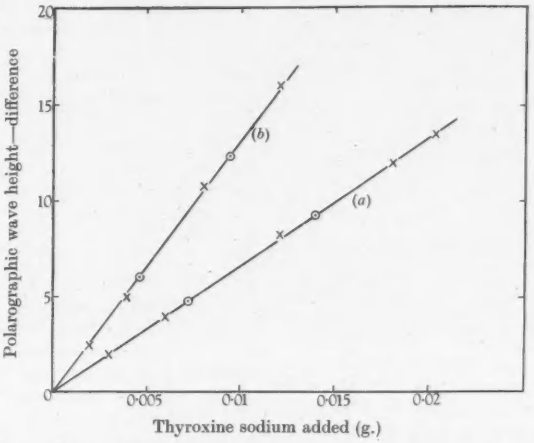


Fig. 2. (*a*) Calibration curve relating thyroxine concentration with polarographic wave height for determination on acid-insoluble fraction from iodinated Ardein. (*b*) Similar curve for iodinated casein fraction.

The percentage of apparent thyroxine sodium in this active fraction is therefore (*a*) 80·6; (*b*) 81·9; average 81·3. The percentage thyroxine sodium content of the active fraction PB 25C as calculated from the iodine content assuming all the iodine present to be thyroxine iodine was 78·2.

Table 3. *Determinations of thyroxine in acid-insoluble fractions of iodinated protein hydrolysates*

Iodinated protein batch no.	Iodine content of active fraction (%)	Thyroxine sodium content calculated on iodine analysis (%)	Thyroxine sodium, polarographic method (%)
Ardein PB4	48·6	76·4	77·2
Ardein PB5	35·3	55·5	50·9
Ardein PB7	38·8	61·0	62·9
Ardein PB10	37·5	58·9	58·78 56·58 56·15 } 57·2
Casein PB12	49·0	77·0	77·2
Ardein PB13	43·5	68·4	73·10 69·30 70·00 } 70·8
Casein PB25	49·7	78·2	81·30 77·40 79·83 } 79·5

A number of active fractions was treated as in the above examples. Thyroxine values were determined for each by the polarographic and iodine methods and the results are given in Table 3.

Thyroxine content of iodinated protein

The amount of acid-insoluble fraction, which was isolated from 100 g. of iodo-protein by the method of separation outlined above, ranged from 0·7 to 1·2 g. Taking the values for thyroxine contents as given in Table 3 then the amount of thyroxine in the original iodo-protein is about 0·6 %. This value agrees well with the amount of thyroxine (0·4 g.) which has actually been isolated from iodinated casein samples by Reineke & Turner (1943).

Thyroxine determination in thyroid powder

An acid-insoluble fraction was obtained from a quantity of thyroid powder. Preliminary work has shown that it is possible to obtain polarographic thyroxine waves in solutions of the acid-insoluble fraction similar to those used in the iodinated protein work. The apparent thyroxine content of this acid-insoluble fraction as determined polarographically was 8·8 % and the thyroxine content as calculated from the iodine content and assuming all the iodine present to be thyroxine iodine was 9·1 %.

DISCUSSION

It is desired to emphasize that the main object in presenting the above observations is to indicate that clear-cut and measurable polarographic waves can be obtained in solutions of an acid-insoluble fraction from iodinated proteins and thyroid powder. If varying amounts of thyroxine are added to these solutions then a linear relationship exists between thyroxine concentration and wave height. If this wave is assumed to be due to thyroxine then the thyroxine content of the acid-insoluble fractions agrees with that derived from the iodine estimations, assuming all the iodine present to be thyroxine iodine. The thyroxine content of these iodinated protein samples on this basis is about 0·6 %. This would indicate that the amount of non-thyroxine iodine compounds in these fractions is rather small, since 0·4 g. of thyroxine has been isolated from 100 g. of iodinated casein. Further work could profitably be done on the effect of any non-thyroxine, acid-insoluble iodine compounds on the polarographic wave of thyroxine.

The polarographic method may be of some immediate interest in the case of thyroglobulin, since in this protein thyroxine is the only known compound containing acid-insoluble iodine (Harington & Randall, 1929).

SUMMARY

1. The baryta hydrolysis of an iodinated protein (100 g.) yields an acid-insoluble active fraction (about 1 g.). A thyroxine value for this fraction can be obtained polarographically. This value agrees fairly closely with that calculated from the acid-insoluble iodine value, assuming all the iodine present to be thyroxine iodine, and is about 0·6 % of the original iodinated protein.

2. A similar agreement between the two thyroxine values was obtained with an acid-insoluble fraction obtained from thyroid powder.

3. In the case of fractions from iodinated protein, non-thyroxine iodine compounds may interfere with the polarographic waves obtained and further work on this may be necessary.

Thanks are due to Messrs J. P. Cavanagh and R. G. Steele for assistance rendered during the course of this work.

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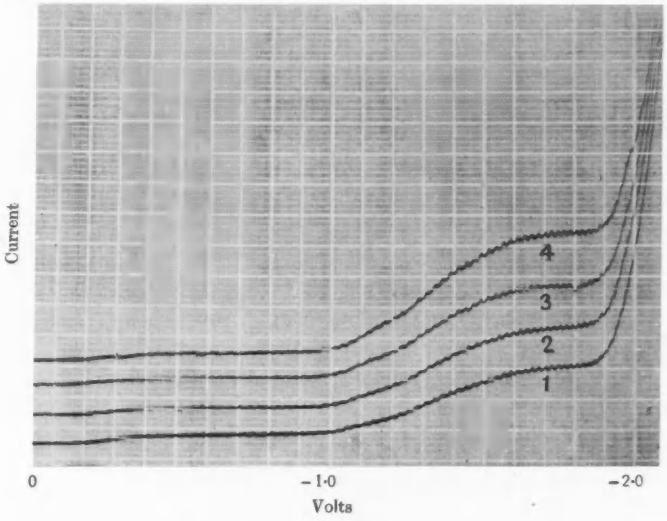

Fig. 1 *a*.

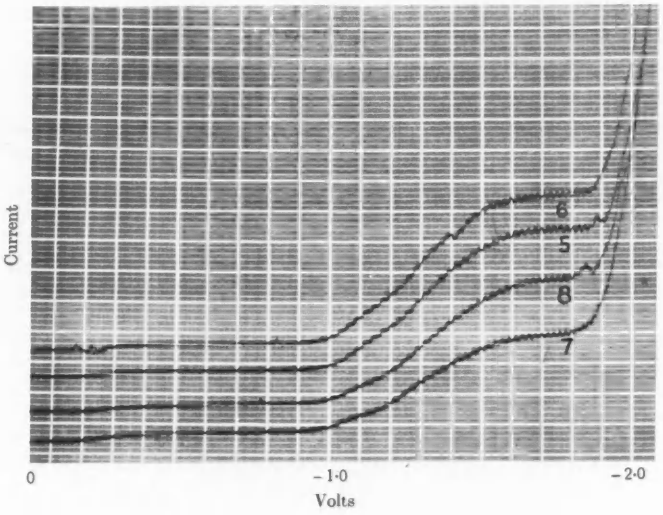

Fig. 1 *b*.

Fig. 1 *a*, *b*. Polarograms of acid-insoluble fraction from iodinated Ardein, without removal of oxygen from test solution. See data in Table 2.

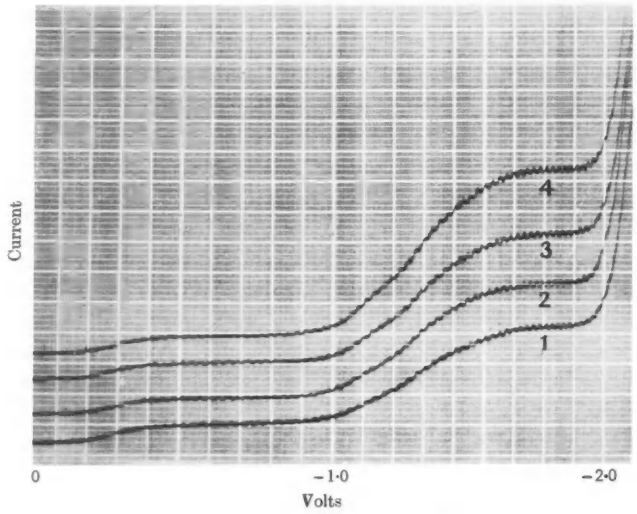

Fig. 3 *a*.

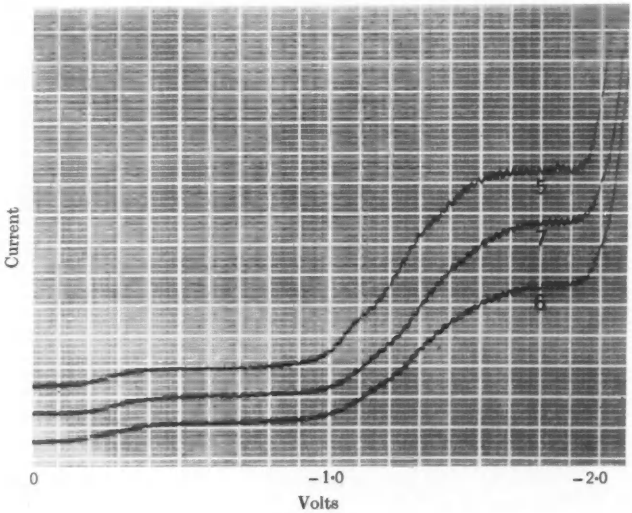

Fig. 3 *b*.

Fig. 3 *a*, *b*. Polarograms of acid-insoluble fraction from iodinated casein without removal of oxygen from test solution. Calibration curve relating thyroxine sodium added, to polarographic wave height is shown in Fig. 2 (*b*).

The Action of Mustard Gas on Ox Cornea Collagen

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The reaction between ox corneal collagen and $\beta:\beta'$-dichlorodiethylsulphide (mustard gas, referred to in the text as *H*), has been studied during work on the effect of various chemical warfare agents on the eye. The aim of the investigation was to establish whether the two substances combined, and, if so, what were the properties of the compound formed, what groups in the protein molecule were involved and whether the compound could be broken up and the normal protein restored. The experiments have shown that *H* combines with collagen under conditions likely to obtain in the eye and that a change in the physical and chemical properties of the treated collagen can be demonstrated.

H causes lesions of the cornea in both man and animals. The histology of small avascular lesions, made by placing small drops of *H* directly on the centre of the rabbit's cornea, has been described by Pullinger & Mann (1943). All cells—the epithelium, the corneal corpuscles and the endothelium—in the path of the liquid are destroyed and subsequently disappear. The collagen fibrils become separated by oedema fluid but do not themselves swell or dissolve, nor are they sloughed out. The collagen fibrils of the cornea make up 97% of the dry weight of the tissue so that a reaction between collagen and *H* to give a product with different properties might be of importance in determining the type of injury. This also holds true for injury of the skin by *H* as collagen is the main protein of the cutis.

In all the experiments reported, fresh collagen that had not been dried at any stage has been used. This was considered important because collagen loses some of its power to swell after being even partly dried. The reaction between *H* and collagen was studied in a neutral solution of 0·9% NaCl at 30° in order to simulate physiological conditions. The amount of *H* which had combined was estimated by determining the rise in sulphur content of the collagen after treatment, and tests of solubility, enzyme digestibility, swelling and stability, etc., were made on both normal and treated collagen preparations and titration curves were made in each case.

METHODS AND RESULTS

Preparation of collagen

Collagen was prepared by the method of Bergmann & Stein (1939). Fresh ox corneas were minced with scissors and suspended in 10% NaCl at 0°. After 24 hr. the corneas were strained off through muslin and minced in a small latapie mincer. The fresh corneas were too tough to go through this mincer, but after the above preliminary extraction the fragments were swollen and were more easily cut. The minced tissue was replaced in fresh 10% saline at 0°. Extraction with saline was continued for a week, with a change of fluid each day, about 5 l. in all being used to extract 100 corneas. The tissue was then extracted with M/15-Na_2HPO_4 at 0° for 3–4 days, the fluid being changed each day, and then with distilled water, until the extract was free from chloride. The wet corneal residue was stored under ether at 0°. This residue was taken to be collagen. There is little elastin in cornea and the remaining corneal constituents are soluble in the extracting fluids. A heavy precipitate of mucin came down on acidifying the early extraction fluids. The residual collagen was much swollen and translucent. After removing as much water as possible by hand pressing between filter paper, the dry weight was found to be 11–14% of the wet weight.

Estimation of sulphur. Known weights of 5–30 mg. of collagen dried over P_2O_5 were incinerated with a perchloric-nitric-copper mixture described by Pirie (1932) and the total sulphur in the incinerate was estimated by the colorimetric method of Cuthbertson & Tompsett (1931). Duplicate incinerations and estimations were done and it was found necessary to do a blank estimation each day.

Reaction of corneal collagen with H

A thick suspension of collagen in 0·9% NaCl was made in a small beaker. Neutral red was added as an internal indicator and the suspension was kept mechanically stirred at 30° in a water-bath. Liquid *H*, 0·05–0·2 ml. was then added and as soon as the indicator changed colour 0·1 N-NaOH was added from a burette to bring the suspension again to neutrality. The reaction was followed until at least 80% of the added *H* had been hydrolyzed, as measured by the total NaOH that had been run in. The collagen was then washed several times with water on the centrifuge until the supernatant fluid was free from chlorine. It was then washed by shaking with several changes of ether to remove residual *H* and stored at 0° under ether. Before analysis, samples were dried *in vacuo* over P_2O_5 and most were then extracted, either with ether or acetone, to remove the last traces of free *H*, or its hydrolysis products.

The rate of hydrolysis of *H* in water depends on the degree of dispersion of the *H*. If it is added as an ethanolic solution hydrolysis is very rapid, but in a purely aqueous medium it is slow and depends on the rate of stirring. Peters & Walker (1923) have shown that NaCl retards hydrolysis. The rate was increased in the presence of collagen but it is difficult to judge whether this was due to

reaction of *H* and collagen or to mechanical break-up of the globules of insoluble *H* by the thick protein suspension. The time for 50% hydrolysis of 0·04 ml. *H* was 180 min. in water and 270 min. in 0·9% NaCl, while 0·05 ml. *H* was 50% hydrolyzed in 75 min. in a thick suspension of collagen in 0·9% NaCl. The reaction was followed by titrating the liberated HCl with 0·1N-NaOH. The suspensions were continuously stirred at 30°.

Table 1 gives the total S contents of different preparations of ox cornea collagen before and after reaction with *H*. Mörner (1899) gives 0·2% S in corneal collagen and Bergmann & Stein (1939) give 0·22% S in tendon collagen.

Table 1. *Sulphur content of ox cornea collagen before and after treatment with H*

Each figure represents the mean of duplicate or triplicate estimations except in sample no. 6.

	Sulphur (%)	
No.	Collagen	*H* collagen
1	0·18	0·63
2	0·18	0·43
3	0·26	0·90
4	0·33	1·0
5	0·20	0·69
6*	0·19	0·46

* Collagen from rat tail tendon; single estimation in each case through lack of material.

Table 1 shows that in every case the total S content has risen after the treatment with *H* but that the rise varies from one preparation to another. It is possible that none of the preparations was saturated with *H*. In preparations 1 and 2, 0·1 ml. *H* was added to 5–10 g. wet collagen and in other preparations which have a higher final S content, 0·2 ml. *H* was used with approximately the same amount of protein. No attempt was made to reach saturation of collagen with *H* as this would probably not occur under natural conditions.

Comparison of the properties of normal and H treated collagen

(*a*) *Swelling.* A characteristic property of collagen is the degree of swelling in acids and alkalis of different strength. Jordan Lloyd (1933) plotted the swelling curves of fresh skin collagen between pH 1 and 12 and found a maximum between pH 2–3. The swelling of normal ox cornea collagen was measured and compared with that of ox cornea collagen after treatment with *H*.

The fragments of collagen and *H*-treated collagen were pressed by hand between filter papers to remove as much water as possible and 0·9 g. samples were weighed into a series of centrifuge tubes. Water, acid or alkali of known strength (10 ml.) was added and the tubes immediately stoppered. After 48 hr. on the bench at 15° with occasional shaking, the supernatant fluid from each tube was pipetted off and its pH determined either by titration or with the glass electrode. The collagen residues were washed once on the centrifuge with water and allowed to drain as completely as possible. The wet weight and the dry weight of each sample was then determined and the degree of swelling taken to be wet weight × 100/dry weight.

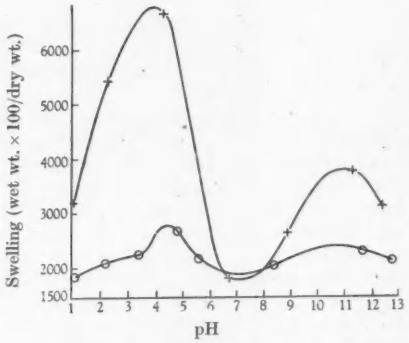

Fig. 1. Swelling curves of normal ox cornea collagen and *H*-treated ox cornea collagen. +—+—+ normal; o—o—o *H*-treated.

Fig. 1 shows that *H*-treated collagen does not swell much either in acid or in alkali. The suppression of swelling is greatest in acid as it is here that the normal protein is most swollen. In neutral solution *H*-treated collagen and normal collagen have their zones of minimum swelling and here both are swollen to about the same extent. A difference in appearance between normal and *H*-treated collagen is discernible at neutrality but becomes much more obvious in acid solution where the normal protein is bluish and translucent and *H* collagen is white and opaque.

(*b*) *Solubility.* Normal collagen is dissolved and forms gelatin when boiled in water. Table 2 shows the proportion of collagen and of *H*-treated collagen converted to gelatin by heating in a boiling water-bath for 8 hr. at pH 4·0 under a simple condenser. The table gives also the S contents of

Table 2. *Conversion of collagen and H collagen to gelatin by treatment with boiling water*

	Collagen		*H* collagen	
No.	Soluble (%)	Insoluble (%)	Soluble (%)	Insoluble (%)
1	82	18	10	90
2	—	—	5	95
3	83	17	10	90
4	69	31	4	96

S content of soluble and insoluble fractions

	S content of collagen (%)			S content of *H* collagen (%)		
No.	Original	Soluble	Insoluble	Original	Soluble	Insoluble
1	0·26	0·09	0·92	0·93	1·07	0·77
2	—	—	—	0·93	0·67	0·94

the soluble and insoluble fractions. The insoluble residues were separated by centrifugation and in preparation 1 both residues and supernatant fluids were frozen and dried over P_2O_5 without further treatment. In preparation 2 the supernatant fluid from *H* collagen was precipitated with ethanol before drying in order to remove any *H* or breakdown products which might have been split off during heating.

H-treated collagen is practically insoluble and judging from the S contents of the soluble and insoluble fractions it is stable under these conditions. It is interesting that most of the S in the normal collagen is contained in the small insoluble fraction.

(*c*) *Enzyme digestibility.* The effect of different proteolytic enzymes was tested by incubating them with the finely chopped collagens in a concentration of 5–20 mg./ml. Samples were taken in a 1·0 ml. all-glass syringe whose tip had been cut off to give a wide opening. If the suspensions were shaken during sampling, fairly good duplicate samples could be taken. These were centrifuged, the insoluble protein was washed once on the centrifuge, dried at 90° and weighed. The loss in weight during incubation with the enzyme was taken as a measure of digestion. Controls showed that no solution of collagen or of *H*-treated collagen took place at the pH used for digestion by papain or cathepsin, but both activated enzymes partly dissolved collagen in one or two of the experiments though not in all. Normal collagen went into solution rather more readily than *H* collagen but the difference is probably insignificant owing to the difficulty of sampling. Some normal collagen went into solution at pH 1·8, the optimum pH for pepsin, but this did not obscure the effect of the enzyme which caused almost complete solution of normal collagen. *H*-treated collagen, in striking contrast to the normal protein, was not digested by pepsin. The activity of the enzymes was tested by the usual methods.

Table 3. *Effect of proteolytic enzymes on collagen and H collagen*

Enzyme	pH	Time	Loss in weight during digestion (%)	
			Collagen	*H* collagen
Trypsin	7·8	48 hr.	0	0
Papain	5·2	24 hr.	12	0
Papain		24 hr.	41	24
Papain		24 hr.	0	10
Papain		3 weeks	44	12
Cathepsin	5·2	24 hr.	38	12
Cathepsin		48 hr.	0	0
Cathepsin		10 days	0	0
Pepsin	1·8	24 hr.	95	18
Pepsin		24 hr.	95	0
Pepsin		24 hr.	90	0
Pepsin		24 hr.	99	18
Pepsin		24 hr.	87	0
Pepsin	4·0	8 days	0	0

Percentage loss in weight of 1·0 ml. samples after digestion at 37°. The enzymes used were: trypsin (0·2%), papain (0·1%) activated with HCN, and pepsin (0·1%) (all British Drug Houses Ltd.). Liver cathepsin (Anson, 1937) was activated by cysteine (Fruton, Irving & Bergmann, 1941).

(*d*) *Stability of H collagen in acid and alkali.* The higher S content, the resistance to pepsin and the insolubility in boiling water at pH 4 of *H*-treated collagen were used as criteria in attempts to detach the *H* residues from the collagen molecule leaving the protein in its original condition. If the reaction between *H* and collagen could be reversed these differences should disappear. In brief, *H* collagen was found to be unstable in dilute alkali where it lost a large part of its extra S, became readily digestible by pepsin and soluble on boiling. Samples of collagen were suspended in 0·02% KOH and left for 24 hr. at room temperature. This seemed to cause some swelling of the *H* collagen but no gross change occurred in either preparation. Merrill (1924) studied the effect of alkali on the hydrolysis of skin collagen and found that none occurred, as judged by absence of N in the supernatant, after 24 hr. at 15° and pH 13.

Table 4. *Effect of alkali on collagen and H collagen*

100 mg. normal and *H* collagen were suspended in 10 ml. 0·02% KOH and left for 24 hr. The sediments were centrifuged down, washed several times and then dried in the frozen state.

(*a*) Effect on S content

Percentage S before KOH		Percentage S after KOH		Change in percentage S	
Collagen	*H* collagen	Collagen	*H* collagen	Collagen	*H* collagen
0·43	0·64	0·29	0·36	−0·14	−0·28
0·32	0·98	—	0·35	—	−0·63

(*b*) Effect on pepsin digestibility

Digestibility (%)			
Before KOH		After KOH	
Collagen	*H* collagen	Collagen	*H* collagen
95	18	95	95

Tests of the pepsin digestibility of *H* collagen held for 24 hr. at 37° in buffers of different pH showed that after incubation at pH 4·7, *H* collagen became 18% digestible, after incubation at pH 7·0 it became 34–45% digestible and after incubation at pH 9·5 it became 99% digestible. A test of the convertibility of *H* collagen to gelatin at different pH ran parallel to the test of pepsin digestibility. At pH 9·5, 85% went into solution but at lower pH little dissolved. The alkaline extracts from *H* collagen gave a positive test for thiodiglycol with selenium dioxide.

Mode of combination of H with collagen

The experiments already reported have shown that combination of collagen with *H* alters its properties considerably. The question arises whether these alterations can give any clue to the mode of linkage of *H* in the treated collagen molecule. The evidence obtainable from the reaction between collagen and other substances, such as formaldehyde, whose point of attachment is known, is meagre. For example Ewald (1919) found that formol-treated collagen was insoluble and was not digested by pepsin, and Hopkins & Wormall (1933) found that gelatin became insoluble after treatment with phenyl isocyanate. In both these cases the free NH_2 groups were blocked by the reagent, and by analogy one might suppose that *H* also has combined with

the free NH_2 groups to give insoluble and non-digestible *H* collagen. On the other hand, the alkali lability of *H* collagen suggests that the reaction is an esterification of the carboxyl groups. Blackburn, Carter & Phillips (1941) found that methylated wool which contained mainly ester methoxyl groups was alkali-labile and gave up its methyl groups at room temperature and pH 9·5.

Further evidence as to the method of linkage of *H* was sought by determination of the titration curve of the normal and treated proteins and a comparison of their staining reactions with acid and basic dyes. The method of determination of the titration curves was essentially the same as the method of determination of the swelling curves with the modification that the acids and alkalis were made up in 0·1 M-KCl as this is thought to give a more uniform penetration of the acid or base into the collagen gel. The preparation of *H* collagen used had a rather low total S content of 0·5%. Normal collagen has 0·2% S, so that 0·30% S in the *H* collagen may have come from *H*. This is a small amount to make a detectable difference in the titration curve and, as expected, the titration curves for normal and *H* collagen were very much alike. *H* collagen appeared to combine with slightly less acid and more alkali than normal collagen but the difference was too small for much reliance to be put on the result. Tests of the staining reactions of normal and *H*-treated collagen with acid and basic dyes showed that, compared with the normal protein, *H* collagen stained less intensely with basic dyes such as methylene blue and methyl violet, and more intensely with acid dyes such as eosin and acid fuchsin. The difference in staining was very clear if small fragments of the collagen were suspended in the dye solutions for a few minutes and then washed once or twice with water. These tests indicate that there was a reduction in the acid groups of collagen as a result of *H* treatment.

A further analysis of an acid hydrolysate of *H* collagen to determine whether any *H* combined with the free NH_2 groups of the molecule has been carried out by Synge & Gordon, using the chromatographic method elaborated by Gordon, Martin & Synge (1943). Acids equally or more readily extractable by the organic solvent than acetyl-alanine would thus be determined. The normal collagen used for analysis had 0·21% S and the *H* collagen 0·69%. Synge & Gordon state no qualitative differences (anomalous bands, etc.) between the normal and *H* collagen were observed during the analysis, and there were no quantitative differences exceeding the experimental error. The procedure would be expected to reveal any reaction of *H* with free NH_2 groups, etc., giving rise to *N*-substituted acid-stable compounds of amino-acids; such compounds after hydrolysis should give acid acetyl derivatives readily extracted by organic solvents. (The main free NH_2 groups in collagen are probably ϵ-amino-groups of lysine residues according to Gurin & Clark (1934).) Thus, while the present work, being negative in outcome, must be interpreted with caution, it suggests that *N* substitution of collagen does not occur during reaction with *H* and that the main reaction is probably an *O* substitution.

On the whole, the evidence is in favour of the view that *H* combines with the free carboxyl groups of collagen although, from the properties of the formol-treated collagen described by Ewald (1919), it would seem that reduction in NH_2 groups also leads to greater insolubility and less ready digestibility by pepsin.

Occurrence of H collagen in vivo

The preparation of a compound of *H* with collagen does not necessarily mean that such combination takes place when *H* burns either the eye or the skin. Some attempts to assess the importance of *H* collagen *in vivo* were made by investigating, first, whether *H* collagen was formed when *H* reacted with whole chopped cornea and, second, whether the tissue reaction to implanted *H* collagen bore any relation to the tissue reaction to an *H* burn of the skin.

Reaction of H with chopped cornea. Fresh chopped ox cornea was suspended in saline and allowed to react with *H* in the usual way. After this treatment 10% NaCl or M/15-Na_2HPO_4 did not extract any corneal mucin, although these solvents remove all mucin from normal cornea. Berenblum (1940) found that after treating skin with *H* very little nucleoprotein could be extracted from it and he considered that the nucleoprotein had been precipitated by the *H* in the skin. The insolubility of the mucin from the cornea may either be due to a change in the permeability of the collagen gel, in whose interstices the mucin is normally held, or it may be due to a reaction between *H* and corneal mucin. No evidence for such a reaction has been obtained and it was found that the mucin could be successfully removed from *H*-treated cornea by digestion with pepsin. Digestion was carried out for 10 days at 37° with several changes of enzyme solution. After this time, as there seemed no further change in the insoluble residue, it was centrifuged, washed repeatedly with water and its properties compared with those of *H* collagen. A dried sample, after ether extraction, gave total S, 0·74% and in general the properties were similar to those of other preparations of *H* collagen. This result shows that *H* combines with collagen in the chopped tissue as well as in a suspension of the purified protein.

Tissue reaction to implantation of normal and H collagen. Implants of normal and of *H* collagen were made in two rabbits. This experiment was done in collaboration with Dr B. D. Pullinger and I am indebted to her for the histological description of the lesions and the tissue reaction to the implants. The rabbits were laboratory stock (crossed Dutch). Two large areas, one on each side of the mid-ventral line, were shaved. The following day the animals were anaesthetized and four small slits made in each of the shaved areas. About 10 mg. (dry) of normal collagen was implanted subcutaneously through each of the four slits on one side of the animal and about 10 mg. (dry) of *H* collagen implanted in the same way on the other side of the animal. The implantations were done with a metal spatula and the small lumps of protein were pushed away from the slit in the skin. The slits were then stitched up. The animals recovered quickly. At first the animals were examined and the appearance of the reactions noted each day; later, weekly examinations were made. At the end of the first and nearly all subsequent weeks a piece of normal and a piece of *H* collagen were removed from one or other of the animals alternately. The animal was anaesthetized and the protein fragment attached to the surrounding skin was cut out and dropped immediately into Zenker's fixative. The loose edges of skin were then stitched together. The sterility of samples of normal and of *H* collagen was tested by aerobic incubation in broth. No turbidity appeared in 48 hr. Sections were also made of the fragments embedded in agar and stained with Heidenhain's haematoxylin and

other bacterial stains. A few colonies of a diphtheroid bacillus were found.

The following is a summary of the histological reactions of the various normal and *H* collagen preparations.

The comparison lies between the tissue reaction to free normal collagen and to free *H* collagen, prepared by peptic digestion of *H*-treated cornea. The differences are quantitative rather than qualitative. They are visible in both macroscopic and microscopic specimens. Swelling and hyperaemia are far greater in the *H*-treated than in the normal specimens. The swelling increases up to 10 days then slowly decreases over a period of several weeks. The microscopic differences are so great that for 5–6 weeks it is possible to distinguish and name the specimens correctly even when they are unlabelled. The implanted masses of *H*-treated and normal collagen are encapsuled within a week by a fibrocellular capsule which soon becomes vascular. The cells of the capsule are mainly fibrocytes, granular leucocytes and lymphocytes. The normal collagen lies in the capsule surrounded by a few granular leucocytes and macrophages, but in the case of the *H* collagen it is surrounded by enormous numbers of these cells. It is this difference in the number of invading cells which enables one to distinguish the two specimens at any time before the sixth week. Many of these cells are ill-preserved at and after 3 weeks owing to karyolysis and to pyknosis of nuclei. From the fourth week onwards, large numbers of a particular kind of macrophage and of lymphocytes (sometimes in nodular form with accompanying reticulum cells) appear within the capsule. These macrophages have abundant foamy cytoplasm containing material which is stainable with van Gieson's solution and with Mallory's aniline blue. At 5 weeks all the free normal collagen, and at 8 weeks all the free *H* collagen has disappeared except for the stainable material, probably ingested collagen, in the macrophages. (No observations were made on the *H* collagen at 6 and 7 weeks; one specimen was lost through dispersal in the loose tissue of the groin; another ulcerated out through a stitch abscess.)

The features worthy of notice in these lesions are: (1) the slow disappearance of normal and *H* collagen (the *H* collagen being slightly the slower of the two); (2) the more intense inflammatory reaction to *H* collagen as shown by greater swelling, hyperaemia and cellular migration.

The second feature indicates that while both normal and *H* collagen cause an inflammatory reaction, the *H* collagen is considerably more poisonous than the normal collagen. The presence of polymorphs points also to a certain degree of solubility of the two collagens. There is probably some chemotactic and sensitizing action. These leucocytes appear to be unable to digest either collagen, yet when the stimulus for their presence has ceased and they have died *in situ*, the collagen becomes ingested by macrophages.

DISCUSSION

The suggestion has been made (Pullinger, 1946) and evidence presented to support it, that the action of *H* on the skin is to cause coagulation necrosis. The outstanding difference between this and other forms of necrosis is the relative resistance of the dead product to solution by tissue cells or fluids. Thus it can lie *in situ* for considerable periods of time. *H* collagen *in vitro* is relatively resistant to solution by enzyme action and according to the experiments recorded it is also insoluble *in vivo* after subcutaneous implantation.

The evidence from the experiments on tissue reaction to implantation is in favour of the view that the insolubility of tissue 'burned' by *H* may in part at least be due to the presence of a compound of *H* and collagen. That such a compound of *H* with collagen can be formed possibly by reaction of *H* with the carboxyl groups is shown by the *in vitro* experiments here recorded.

Evidence that *H* remains fixed in the cornea has also been obtained through the use of *H*-containing radioactive sulphur. Hamilton & Axelbrod (1943), describing the detection of radioactive *H* in tissue sections, showed that considerable amounts of *H* were detectable in rabbit cornea 7 days after the cornea had been exposed to *H* vapour. The *H* was distributed throughout the whole thickness of the cornea, showing that it had combined with some insoluble corneal constituent. Other substances, such as benzoylchloride and *o*- and *p*-chlorobenzylchlorides which have been shown by Medveczky & Uhrovits (1931) and by Landsteiner & Jacobs (1936) to form compounds with proteins also cause coagulation necrosis of mouse skin (Pullinger, 1946), although the tissue is not so deeply affected as it is by *H*.

There is, of course, ample evidence that there are many other ways in which *H* may damage the skin and the cornea, e.g. by inhibition of specific enzymes as described by Dixon & Needham (1946). It has been the purpose of this paper to define one possible reaction between *H* and collagen and to determine whether the properties of the reaction product fit it to play a part in the pathology of the *H* lesion.

SUMMARY

1. The reaction between ox corneal collagen and $\beta:\beta'$-dichlorodiethylsulphide (*H*) has been studied.

2. *H* combines with collagen to give a product with a higher S content. This compound, unlike normal collagen, swells only slightly in acid solution, is resistant to pepsin digestion, and is insoluble when boiled in dilute acid.

3. The mode of combination of *H* with collagen is discussed.

4. The tissue reaction to implanted *H* collagen is described and it is concluded that some of the characteristics of an *H* 'burn' of the skin or eye may be due to the presence of *H* collagen in the injured tissue.

This work was done between 1940 and 1944, partly at the Laboratories of the Imperial Cancer Research Fund, Mill Hill, and partly at Oxford. I wish to thank the Director of Scientific Research (Defence), Ministry of Supply, for permission to publish the results. It is also a pleasure to thank Dr W. E. Gye for his help and interest, Dr Pullinger for carrying out the experiments on implantation of collagen, and Dr R. L. M. Synge and Dr H. Gordon for analyzing *H* collagen.

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The Determination of Dienoestrol

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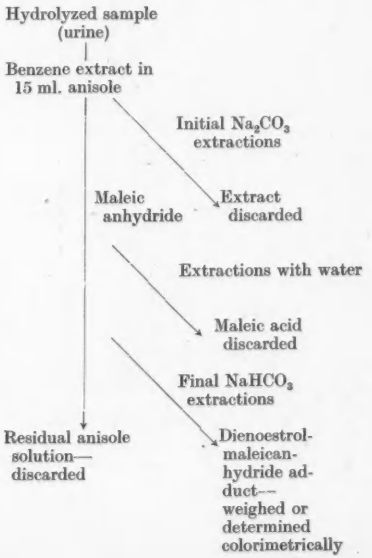
Since the clinical and veterinary use of synthetic oestrogens has become so general it is important to devise a simple routine method for the determination of these substances, particularly in biological samples. It would also be an advantage to possess a method applicable at the same time to pharmacological preparations.

Many attempts have been made in this direction, references to which are given by Malpress (1945) but it is probably fair to state that none is applicable to samples of synthetic oestrogens containing biological contaminants. The colouring matter in urines for example, interferes with colorimetric methods, e.g. the Dingemanse (1939) antimony pentachloride method (Warren & Goulding, 1945) and the presence of other phenols interferes with methods dependent on nitration (Malpress, 1945). No method has yet been evolved which will overcome these difficulties by quantitative separation of the synthetic oestrogen or a derivative.

The present communication describes a method of separating a derivative of dienoestrol in about 70% efficiency from most other contaminants likely to be found in biological samples. It makes use of the facts that dienoestrol is a diene and reacts with maleic anhydride and that the resultant adduct is soluble in aqueous sodium bicarbonate solution, (Wilder Smith, 1946). The method may be represented diagrammatically as follows:



The only other reactions likely to occur besides the dienoestrol-maleic anhydride addition, are (1) reaction with any other suitable conjugated dienes present, and (2) reaction with alcohols to form half esters. Both (1) and (2) would give new bicarbonate soluble substances. From the fact that the method does give a small blank result with urines containing no dienoestrol, it appears that either (1) and/or (2) do in fact take place to some extent. Indeed, if the urine extract-maleic anhydride reaction mixture is heated at 100° for 30 min. a blank of several mg./l. of normal urine can be obtained. If, however, the reaction is carried out at room temperature, the blank formed amounts to approximately 1 mg./l. normal urine extract. It has been found possible to remove from this blank, by a short steam distillation, most substances interfering with the colour reaction described later. The dienoestrol-maleic anhydride adduct, after hydrolysis to the dicarboxylic acid, is non-volatile in steam. This hydrolysis occurs during the sodium bicarbonate extraction.

METHOD

For the preliminary work, dienoestrol in varying amounts was added to a benzene extract corresponding to 1 l. of hydrolyzed normal male urine, thus evading the time-consuming continuous benzene extraction for each determination. In all the later experiments anisole was added to the benzene extract and the benzene removed by distillation, leaving an anisole solution of the extracted material, though results can be obtained by working directly on the concentrated benzene extract, provided the dienoestrol concentration is not high (over 10 mg./l. urine).

(*a*) *For concentrations of dienoestrol not below 2–5 mg./l. urine*

(1) *Initial bicarbonate extractions.* The urine sample (1–2 l.) is hydrolyzed by refluxing for 10 min. after addition of 100 ml. concentrated hydrochloric acid/l. urine. The urine is extracted for 24 hr. in a continuous benzene extractor, and the benzene extract concentrated by distillation from an oil bath into 10 ml. anisole. After removal of all the benzene, the volume of residue is made up to 15 ml. with anisole and the anisole solution transferred to a 35 ml. cylindrical shaking flask (fitted with a ground glass stopper held in position by lugs and small springs) and agitated 1–5 min. with 15 ml. of a 10% solution of aqueous sodium carbonate. After removal of the lower sodium carbonate layer, which is discarded, the process is repeated until less than 0·5 mg. of acid material is extracted by the carbonate from the anisole layer; usually 5–7 extractions are required. If desired these extractions may be carried out by hand. It is usually necessary to warm the anisole layer in warm water to effect rapid separation of the two layers.

The weight of acid material in the carbonate layer is determined as follows: After two hand extractions with 25 ml. portions of ether (to remove any material taken over mechanically from the anisole layer) the carbonate layer is made acid to congo-red paper by adding concentrated hydrochloric acid, then extracted four times with 25 ml. portions of ether and the ether dried over anhydrous sodium sulphate. Evaporation of the ether into a small weighed flask followed by vacuum desiccation gives the weight of acid material extracted by the carbonate. The weight may be checked by titration against 0·01 N-alkali. However, the weighing and vacuum desiccation may be dispensed with by following the course of the extraction by titration alone. Since the oil thus extracted is not rapidly soluble in dilute alkali it is convenient to dissolve it in a few ml. of acetone before titration, then to titrate the acetone solution. Thymol blue has been found to be a suitable indicator for use in the presence of the brown-coloured solutions obtained. With experience, the determination of the acid material in the carbonate layer can be dispensed with, the colour of the carbonate extract being a sufficient guide.

(2) *Reaction with maleic anhydride.* The anisole solution thus exhaustively extracted with sodium carbonate is now ready for reaction with maleic anhydride. Solid maleic anhydride (1·0 g.) is added, and the solution agitated at room temperature overnight. Excess maleic anhydride is then removed by extracting with water in a similar manner to the initial carbonate extractions—15 ml. distilled water are added, the solution shaken for 10 min. and the water layer separated. Here again, warming is an aid to the rapid separation of the anisole and aqueous layers. The process is repeated with short hand washings until the extraction is complete as ascertained by titration of the separated water layer against 0·01 N-alkali; usually 5–6 water washings are sufficient.

(3) *Final sodium bicarbonate extraction.* The anisole solution thus exhaustively extracted with water is now extracted with 15 ml. saturated aqueous bicarbonate overnight, followed by two 15 ml. bicarbonate portions for 2 hr. each. The combined bicarbonate layers are made acid to congo red, with dilute hydrochloric acid, steam distilled for 30 min., the steam distillate being discarded, and then extracted with ether, and dried over sodium sulphate. The steam distillation removes the sodium bicarbonate soluble substances in the blank which interfere with the colour reaction described in the next section. After removal of the ether by distillation from a small weighed flask, the residue is vacuum-desiccated and weighed. If x is the weight of adduct recovered, then the original weight of dienoestrol = $266x/384$.

Experimental errors. Some losses of dienoestrol obviously occur during the preliminary extractions with sodium carbonate and water, but since the recoveries reached are approximately 60–70% of added dienoestrol where 3–20 mg./l. urine were present, they can be allowed for.

A more serious error becomes apparent when the dienoestrol concentration in the urine falls below about 3 mg./l. since there is a final blank yield of 0·8–1·2 mg./l. of acid-hydrolyzed urine. It is possible to reduce this blank when acidified, but unhydrolyzed, urines are used, but the method is obviously unsuitable where conjugated dienoestrol is present. The difficulty has been overcome by making use of the fact that the dienoestrol-maleic anhydride adduct couples with diazotized sulphanilic acid to give a red dye with absorption in the 480–510 mμ band. The blank gives little colour on coupling with diazotized sulphanilic acid provided the steam distillation previously referred to is carried out, so that by use of the Spekker absorptiometer,

the blank is eliminated and at the same time the method is made sensitive to less than 500 μg. total dienoestrol present.

(b) For concentrations of dienoestrol below 2 mg./l. urine: application of Spekker absorptiometer

The urine is extracted and worked up exactly as under (*a*) but owing to the blank, the final weighing will give a high result. The final material is therefore made up to 5 ml. with 2N-NaOH and 1 ml. of it coupled with 2 ml. of diazotized sulphanilic acid; after 5 min. at room temperature the solution is diluted with 10 ml. N-NaOH; after a further 5 min. the Spekker reading is taken. Ilford spectrum green filters (510–620 mμ) are used. From a graph constructed from readings with known additions of adduct, the amounts present in unknown samples may be determined.

Table 1. *Recovery of dienoestrol added to normal male urine*

Sample	Weight of dienoestrol added (mg./l.)	Weight of dienoestrol found (mg./l.) Gravimetrically	Colorimetrically
1	77·8	62·2	—
2	60·0	42·2	—
3	8·33	9·5	7·5
4	6·5	5·4	8·0
5	5·5	4·1	5·9
6	5·0	5·8	5·5
7	5·0	4·8	5·0
8	4·0	5·3	3·1
9	4·0	4·1	4·0
10	4·0	2·1	3·4
11	3·33	3·2	2·3
12	3·33	2·1	2·3
13	3·0	4·8	3·8
14	2·0	1·1	1·0
15	1·49	1·8	0·8
16	1·0	1·6	0·6
17	1·0	1·6	0·6
18	0·4	1·8	0·4
19	0·2	1·7	0·2
20	0·2	0·9	0·2
21	0·1	1·1	0·1
22	0·1	1·1	0·2
23	0·0	0·7	0·0
24	0·0	0·6	0·0
25	0·0	1·5	0·0

Preparation of diazobenzenesulphonic acid solution. 1·0 g. sulphanilic acid is dissolved in 8·0 ml. N-NaOH and 50 ml. distilled water added. The solution is then acidified with 7·0 ml. 36% (w/w) hydrochloric acid and cooled to 0° in ice. 8·0 ml. 10% sodium nitrite solution at 0° are added, the solution made up to 100 ml. with distilled water. 2·0 ml. of this solution are used for each determination.

Preparation and properties of dienoestrol-maleic anhydride adduct (4:5-di-(p-hydroxyphenyl)-3:6-dimethyl-Δ^4-tetrahydrophthalic anhydride)

2·66 g. dienoestrol (0·01 mol.) and 1·96 g. maleic anhydride (0·02 mol.) are refluxed together for 5 min. in 150 ml. benzene with just sufficient acetone added to effect solution, and then concentrated to one-third volume and set aside to crystallize. The crystalline product contains one molecule of benzene of crystallization which is readily lost on exposure to the atmosphere. After four crystallizations from benzene and acetone m.p. 197–200° (yield: 95%). (Found: C, 72·4; H, 5·6. $C_{22}H_{20}O_5$ requires C, 72·6; H, 5·5%.)

RESULTS

Table 1 shows the results of several determinations of dienoestrol added to normal male urine and Fig. 1 shows the variation of Spekker reading with concentration of dienoestrol-maleic anhydride adduct.

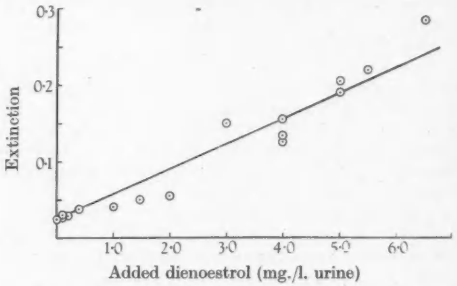


Fig. 1. Colour development of dienoestrol-maleic anhydride adduct with diazotized sulphanilic acid.

SUMMARY

A method for the determination of dienoestrol in urine has been evolved depending on the formation of its adduct with maleic anhydride. The adduct may be recovered and weighed, or determined colorimetrically, according to the amount present. High recoveries of added dienoestrol over a range of 0·2–80 mg./l. are obtained.

The preparation and properties of the adduct are briefly described.

We are indebted to the Council of the Middlesex Hospital Medical School and to Prof. Dodds for the provision of laboratory facilities, and to the Medical Research Council for a grant for our assistant Mr B. D. Shepherd.

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The Separation of Non-ketonic and Ketonic Sodium Glucuronidates from the 'Sodium Pregnanediol Glucuronidate' of Human Pregnancy Urine: the Isolation of Pure Sodium Pregnane-3(α), 20(α)-diol Glucuronidate*

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It has recently been shown (Marrian & Gough, 1946 *a*, *b*) that the 'sodium pregnanediol glucuronidate' ('NaPG') prepared from human pregnancy urine by the method of Venning & Browne (1936) contains about 20% of a water-soluble derivative of pregnan-3(α)-ol-20-one which cannot be removed by repeated recrystallization from 90% (v/v) aqueous ethanol or by repeated precipitation from 50% (v/v) aqueous acetone with 10 volumes of dry acetone. The assumption was made that this pregnanolone derivative is probably the sodium salt of the glucuronide.

Since pure sodium pregnane-3(α), 20(α)-diol glucuronidate was required in connexion with other work in progress in this laboratory, it was decided to attempt the separation of this substance from the 'NaPG' mixture and at the same time to attempt the isolation of the hitherto undescribed sodium pregnan-3(α)-ol-20-one glucuronidate, the presence of which had been assumed.

RESULTS

At the outset various possible methods of separating the ketonic from the non-ketonic glucuronidates were considered and tested experimentally, using a qualitative Zimmermann test (procedure of Callow, Callow & Emmens, 1938) on 5 mg. quantities to detect the presence or absence of ketonic material in the fractions obtained.

Partition chromatography of the glucuronidic acids obtained by acidification of an aqueous solution of 'NaPG', on a potato starch column in moist butanolic solution (cf. Elsden & Synge, 1944) resulted in no separation of the ketonic and non-ketonic components.

By repeated recrystallization from ethanol of the 'triacetate lactone' obtained from 'NaPG' by acetylation at room temperature (Heard, Hoffman & Mack, 1944) a fraction was obtained which gave a less intense Zimmermann reaction than did the original triacetate lactone, but the losses of material at each crystallization were so great and the degree of purification effected so small that it was evident that the method would be of little value for the preparation of pure sodium pregnanediol glucuronidate in large quantities.

Separation of ketonic and non-ketonic glucuronidic acids by means of Girard's reagent T

Since it is possible to extract with ether the glucuronidic acids from an acidified solution of their sodium salts, the possibility was investigated of separating the ketonic and non-ketonic glucuronidic acids by means of trimethylammoniumacetohydrazide chloride (Girard's reagent T) according to the method of Girard & Sandulesco (1936). This method for the separation of ketonic from non-ketonic substances involves forming the trimethylammoniumacetohydrazone of the ketonic component by boiling the material with the reagent in 10% (w/w) ethanolic acetic acid, followed by ether extraction of the non-ketonic component after neutralization of nine-tenths of the acetic acid and dilution with water. In preliminary trials of the method, in which the reagent T was omitted, it was found that the glucuronide linkage in 'NaPG' is stable under the conditions used for forming the hydrazone. However, it was not found to be possible to extract with ether the pregnanediol glucuronidic acid after neutralization of nine-tenths of the acetic acid, and it was therefore concluded that at the pH attained the pregnanediol glucuronidic acid is largely ionized. Accordingly the possibility of effecting the separation, omitting the partial neutralization of the acetic acid, was investigated.

Working under ice-cold conditions and as rapidly as possible in order to minimize the hydrolysis of the trimethylammoniumacetohydrazone, a 'non-ketonic' fraction was obtained which yielded, after two further successive separations in the same manner, a final product which gave a virtually negative Zimmermann reaction. After purification and drying at 137° a sodium salt, analyzing in good

* A preliminary account of this work was given to the Biochemical Society on 27 September 1946 (see *Biochem. J.* 40, xi).

agreement with the formula $C_{27}H_{43}O_8Na$, was obtained (m.p. 283·5–284·5° corr., decomposition and evolution of gas). This gave a strongly positive Tollens reaction for glucuronic acid. The purified product was obtained in a yield of 28 % of the original 'NaPG' or 35 % of theory on the assumption that the 'NaPG' had contained 80 % of non-ketonic glucuronidate. The low yield can probably be ascribed to incomplete extraction of the glucuronidic acid from aqueous solution with ether.

The 'ketonic' fractions from each of the three successive Girard separations were recovered as their sodium salts, combined and subjected to two further Girard separations, the non-ketonic fractions obtained in each being discarded. The sodium salt of the final ketonic fraction yielded after purification a product which melted at 257–260° (corr., with decomposition and evolution of gas), gave an intense brick-red colour in the Zimmermann test, and a strongly positive Tollens reaction. Analysis suggested, however, that it was contaminated with inorganic material. The yield was 6 % of the original 'NaPG'.

The efficiency of the whole separation process and the purity of the products obtained was investigated by determining quantitatively the pregnanediol and 20-ketosteroid contents of the toluene-soluble material obtained from the original 'NaPG', and the non-ketonic and ketonic sodium glucuronidates after acid hydrolysis. The methods used for these determinations were those described by Marrian & Gough (1946 *b*). It should be noted that these methods are not highly accurate. The results are shown in Table 1.

Table 1. *Analysis of glucuronidate fractions*

Toluene-soluble hydrolysis product from	Pregnanediol content (%)	20-Ketosteroid content (%)	M.p. (corr.) without purification
Original sodium pregnanediol glucuronidate	77	23	211–220°
Non-ketonic sodium glucuronidate	98	5·2	233–237°
Ketonic sodium glucuronidate	23	85	127–147°

The pregnanediol and 20-ketosteroid contents of the unpurified hydrolysis product obtained from the non-ketonic sodium glucuronidate indicate that this material is nearly pure pregnane-3(α), 20(α)-diol, a conclusion borne out by the melting-point, which was raised after recrystallization from ethanol to 236–238° (not depressed by admixture with an authentic sample of pregnane-3(α), 20(α)-diol).

These results, considered together with the results of the elementary analyses referred to previously, clearly show that the non-ketonic sodium glucuronidate obtained is nearly pure sodium pregnane-3(α), 20(α)-diol glucuronidate. There are grounds for believing that this substance may not have been previously isolated in a state approximating to purity from human pregnancy urine.

In confirmation of the results obtained by Marrian & Gough (1946 *b*) with impure 'NaPG' it has been found that sodium pregnanediol glucuronidate takes up $3H_2O$ in moist air, and that this trihydrate is converted to the anhydrous substance at 137° *in vacuo* over P_2O_5, whilst *in vacuo* over $CaCl_2$ at room temperature it loses $1·5H_2O$.

The hydrolysis product obtained from the ketonic sodium glucuronidate is not pure pregnan-3(α)-ol-20-one, since the melting-point before purification is low and since it gives a colour with concentrated H_2SO_4 corresponding to a pregnanediol content of about 20 %. This contaminant may be pregnanediol itself or some ketonic substance, which, unlike pregnan-3(α)-ol-20-one, is strongly chromogenic in the H_2SO_4 reaction but does not give a Zimmermann reaction. The ketonic sodium glucuronidate obtained is clearly not a pure substance, although it is probably largely composed of sodium pregnan-3(α)-ol-20-one glucuronidate, since the melting-point of the hydrolysis product was raised by purification to 145–148·5° (corr.) (not depressed by admixture with authentic pregnan-3(α)-ol-20-one). Attempts at the further purification of this ketonic sodium glucuronidate fraction are now in progress.

Pregnane-3(α), 20(α)-diol glucuronidic acid was obtained from the purified sodium salt and, after purification, melted at 178–180° (corr., with decomposition and evolution of gas), and had $[\alpha]_D^{20·5°} - 5·0 \pm 0·5°$. The acid which had been stored in a moist atmosphere lost $1·5H_2O$ *in vacuo* over $CaCl_2$ at room temperature, and analytical figures were then obtained in good agreement with those required for the anhydrous glucuronidic acid, $C_{27}H_{44}O_8$.

EXPERIMENTAL

Separation and purification of the non-ketonic sodium glucuronidate from 'NaPG'

'NaPG' (1·050g.) (m.p. 280°; corr., with decomposition and evolution of gas) was dissolved in 50 ml. of 10 % (w/w) ethanolic acetic acid and heated under reflux in a boiling water-bath for 1 hr. with 3 g. trimethylammoniumacetohydrazide chloride. After cooling under the tap and then in a freezing mixture, the solution was transferred to a chilled separating funnel with 500 ml. of chilled water and 250 ml. of chilled ether. After shaking, the aqueous layer was run off and re-extracted seven times as rapidly as possible with 250 ml. portions of chilled ether. The ethereal extracts were combined and extracted once with 100 ml. of 0·5 N-NaOH and three times with 300 ml. portions of 0·1 N-NaOH. The combined NaOH extracts were then extracted six times with a total volume of 2 l. butanol and the combined butanolic extracts allowed to stand in a

separating funnel until completely clear. After running off the small aqueous layer that separated, the butanolic extract was saturated with CO_2 and evaporated to dryness under reduced pressure.

The material so obtained was subjected to two further 'Girard' separations in succession in a similar manner. In these separations, however, the amount of trimethylammoniumacetohydrazide chloride was reduced to 2·5 g. and the volume of ethanolic acetic acid to 30 ml. The volumes of water, ether and NaOH solution, but not of butanol, were reduced proportionately. The final butanolic extract obtained after the third separation was washed twice with 1/12 volume of water before evaporating to dryness. A yield of 0·436 g. of crude non-ketonic sodium glucuronidate was obtained. This was dissolved in 20 ml. of 50% (v/v) aqueous acetone and precipitated by the addition of 200 ml. of dry acetone. The precipitate was filtered with suction and washed with dry acetone. After a second precipitation by 10 volumes of dry acetone from solution in 16·5 ml. of 50% acetone a product weighing 0·305 g. was obtained.

This material was combined with 0·344 g. of a second batch of purified non-ketonic sodium glucuronidate obtained from 0·986 g. of 'NaPG' by the same procedure. The combined batches were dissolved in 34 ml. of 50% acetone and precipitated with 340 ml. of dry acetone. The precipitate was collected in the usual way and dried at room temperature *in vacuo* over $CaCl_2$. A total yield of 0·572 g. from 2·036 g. 'NaPG' was obtained. This purified non-ketonic sodium glucuronidate melted at 283·5–284·5° (corr., with decomposition and evolution of gas) and gave a virtually negative Zimmermann reaction on 5 mg. It gave a strongly positive Tollens reaction for glucuronic acid.

Weight after exposure to moisture-saturated atmosphere for 15 hr., 0·1735 g. (*a*). Weight after drying to constant weight *in vacuo* over $CaCl_2$ at room temp., 0·1649 g. (*b*). Weight after drying for 10 hr. *in vacuo* over P_2O_5 at 137°, 0·1568 g. (*c*). Weight after re-exposure to moist atmosphere for 24 hr., 0·1741 g. (*d*). Weight after redrying to constant weight *in vacuo* over $CaCl_2$ at room temp., 0·1641 g. (*e*). Moisture content of $CaCl_2$ dried substance $=(b-c)/b \times 100 =$ 4·9; $(e-c)/e \times 100 = 4{\cdot}4\%$. Moisture content of fully hydrated substance $=(a-c)/a \times 100 = 9{\cdot}6$; $(d-c)/d \times 100 =$ 9·9%. Calc. for $C_{27}H_{43}O_5Na$, $1{\cdot}5H_2O$; 4·9% H_2O; $C_{27}H_{43}O_5Na$, $3H_2O$: 9·4% H_2O. 2·389 mg. dried at 137° for 10 hr. *in vacuo* over P_2O_5 gave 5·319 mg. CO_2, 1·853 mg. H_2O, 0·253 mg. Na_2CO_3 and 0·02 mg. unburned C. Found: C, 62·7; H, 8·7; Na, 4·6. Calc. for $C_{27}H_{43}O_5Na$: C, 62·5; H, 8·4; Na, 4·4%.

Separation of the ketonic sodium glucuronidate from 'NaPG'

The ketonic fractions from each of the three separations with trimethylammoniumacetohydrazide chloride described in the preceding section were obtained in the following manner: the aqueous phase after removal of the non-ketonic fractions by ether extraction was acidified with concentrated HCl (2·0 ml./100 ml. solution). After standing at room temperature for 1 hr., the mixture was made just neutral to litmus by the addition of 10N-NaOH, and then an excess of 1·0 ml. of 10N-NaOH was added/100 ml. This alkaline solution was extracted six times with a total volume of 1 l. butanol, and the extract allowed to stand in a separating funnel until clear. The small aqueous layer that separated was run off and the butanol evaporated to dryness after saturating with CO_2.

The three ketonic fractions so obtained were combined and subjected to two further successive separations with trimethylammoniumacetohydrazide chloride. The reactions were carried out as previously described in 30 ml. of 10% (w/w) ethanolic acetic acid with 2·5 g. of the reagent. The non-ketonic fractions obtained from these separations were rejected. The butanolic extract containing the ketonic fraction from the last separation was washed twice with 1/12 volume of water and evaporated to dryness under reduced pressure. The crude ketonic fraction thus obtained was dissolved in 15 ml. of 50% (v/v) aqueous acetone and precipitated with 150 ml. of dry acetone. After a second precipitation from 10 ml. of 50% acetone with 10 volumes of dry acetone a yield of 0·115 g. of ketonic sodium glucuronidate was obtained.

This material was combined with 0·063 g. of a second batch of ketonic sodium glucuronidate obtained from 0·986 g. of 'NaPG' by the same procedure. The combined batches were given a final purification by dissolving in 15 ml. of 50% acetone, filtering and then precipitating with 150 ml. of dry acetone. From 2·036 g. 'NaPG', 0·131 g. of ketonic sodium glucuronidate melting at 257–260° (corr., with decomposition and evolution of gas) was obtained. The product gave an intense brick-red colour in the Zimmermann reaction and a strongly positive Tollens test for glucuronic acid.

2·470 g. dried at 137° for 10 hr. *in vacuo* over P_2O_5 gave 5·201 mg. CO_2, 1·782 mg. H_2O, and 0·333 mg. Na_2CO_3. 1·994 mg. similarly dried gave 4·193 mg. CO_2, 1·449 mg. H_2O, and 0·271 mg. Na_2CO_3. Found: C, 58·9 and 58·8; H, 8·1 and 8·1; Na, 5·8 and 5·9. Calc. for $C_{27}H_{41}O_5Na$: C, 62·7; H, 8·0; Na, 4·5%.

Hydrolysis of 'NaPG' *and of the separated non-ketonic and ketonic sodium glucuronidates*

Samples of approximately 100 mg. each of the original 'NaPG' of the non-ketonic sodium glucuronidate and of the ketonic sodium glucuronidate were each treated as follows. The sample was suspended in 250 ml. of water and 65 ml. of toluene added. The mixture was heated to boiling under reflux and then 25 ml. of conc. HCl added. Boiling was continued for 10 min. and the flask then cooled under the tap. The mixture was transferred to a separating funnel, shaken thoroughly and the aqueous layer run off. This was re-extracted four more times with 65 ml. portions of toluene. The combined toluene extracts were washed once with 50 ml. of 5% (w/v) $NaHCO_3$, twice with 50 ml. portions of water, and evaporated to dryness.

Pregnanediol was determined in the hydrolysis products by the Talbot, Berman, MacLachlan & Wolfe (1941) procedure as described by Marrian & Gough (1946*b*). 20-Ketosteroids were determined as described by the latter authors.

'NaPG' (99·8 mg.) yielded 54·2 mg. of toluene-soluble hydrolysis product melting at 211–220° (corr.). 0·254 mg. of this product gave a colour in the H_2SO_4 reaction equivalent to the colour given by 0·197 mg. of authentic pregnane-3(α), 20(α)-diol. 1·016 mg. gave a colour in the Zimmermann reaction equal to that given by 0·239 mg. of authentic pregnan-3(α)-ol-20-one.

The non-ketonic sodium glucuronidate (103·2 mg.) yielded 62·0 mg. of toluene-soluble hydrolysis product melting at 233–237° (corr.). 0·243 mg. gave the same intensity of colour in the H_2SO_4 reaction as 0·239 mg. of authentic pregnanediol, while 1·943 mg. gave the same intensity of colour in the Zimmermann reaction as 0·102 mg. authentic pregnanolone. After one recrystallization from ethanol the melting-point was 236–238° (corr.) and this was not depressed by admixture with authentic pregnane-3(α), 20(α)-diol (m.p. 235–237°, corr.).

From 101·4 mg. of the ketonic sodium glucuronidate there were obtained 62·0 mg. of toluene-soluble hydrolysis product melting at 127–147° (corr.). In the H_2SO_4 reaction 1·486 mg. gave a colour equivalent to that given by 0·342 mg. of pregnanediol, and in the Zimmermann reaction 0·239 mg. gave a colour equivalent to that given by 0·202 mg. of pregnanolone. The product was purified by adsorption from benzene solution on an Al_2O_3 (6 × 1·3 cm.) column and elution with ether. The product so obtained after two crystallizations from *n*-hexane melted at 145–149° (corr.). Mixed with authentic pregnan-3(α)-ol-20-one (m.p. 147–149·5°, corr.) the melting point was 146·5–149·5° (corr.).

Pregnane-3(α), 20(α)-diol glucuronidic acid from its sodium salt

The purified non-ketonic sodium glucuronidate (0·173 g.) was dissolved in 60 ml. of moist butanol with warming. The solution was acidified with 2 ml. of glacial acetic acid, transferred to a separating funnel, washed three times with 5 ml. portions of water, and finally evaporated to dryness under reduced pressure. The residue was heated to boiling with 5 ml. of water and the mixture filtered hot. The insoluble material was leached twice more in the same way with 5 ml. portions of hot water and then dissolved in 20 ml. of hot 25% (v/v) aqueous ethanol. The hot solution was filtered, evaporated under reduced pressure to the point of incipient crystallization and then set aside in the refrigerator overnight. The well-formed but slightly buff-coloured crystals that separated were collected by centrifugation, washed once in the centrifuge tube with 10 ml. of cold water and dried at room temperature over P_2O_5 *in vacuo*. A yield of 129 mg. of material melting at 178–180° (corr., with decomposition and evolution of gas) was obtained.

Material weighed after exposure to a moist atmosphere (2·160 mg.) was dried to constant weight *in vacuo* over $CaCl_2$ at room temperature. Weight loss observed, 0·122 mg. or 5·6%. Similarly treated, 10·497 mg. lost 0·581 mg. or 5·5%. Calc. for $C_{27}H_{44}O_8$, $1·5H_2O$: 5·2% H_2O. The m.p. was unchanged after drying.

A solution of the anhydrous substance (9·916 mg.) in 0·50 ml. ethanol gave a rotation of −0·049° (standard error 0·0054°) in a 0·5 dm. tube. Hence $[\alpha]_D^{20·5°} = -5·0 \pm 0·5°$.

2·038 mg. dried at room temperature over $CaCl_2$ *in vacuo* for 70 hr. gave 4·866 mg. CO_2 and 1·655 mg. H_2O. 2·378 mg. similarly dried gave 5·688 mg. CO_2 and 1·923 mg. H_2O. Found: C, 65·1 and 65·2; H, 9·1 and 9·1. Calc. for $C_{27}H_{44}O_8$: C, 65·3; H, 8·9%.

SUMMARY

1. 'Sodium pregnanediol glucuronidate' prepared from human pregnancy urine has been separated into non-ketonic and ketonic sodium glucuronidate fractions by means of trimethylammoniumacetohydrazide chloride.

2. The non-ketonic sodium glucuronidate appears to be nearly pure sodium pregnane-3(α), 20(α)-diol glucuronidate, and is contaminated with traces only of ketonic material.

3. Sodium pregnane-3(α), 20(α)-diol glucuronidate dried under different conditions is obtained anhydrous or with 1·5 or 3 molecules of water of crystallization.

4. Pregnane-3(α), 20(α)-diol glucuronidic acid has been obtained from its sodium salt. Under different conditions of drying it is obtained anhydrous or with 1·5 molecules of water of crystallization.

5. The ketonic sodium glucuronidate obtained appears to consist mainly of the hitherto undescribed sodium pregnan-3(α)-ol-20-one glucuronidate; it has not, however, so far been obtained in a pure state.

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Effect of Various Salts on the Synthesis of Ascorbic Acid and Carotene in Cress Seedlings

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The excessive application of nitrogen to apple trees has been shown to reduce the ascorbic acid content of the fruit (Kessler, 1939). A similar observation on grape-fruit was made by Jones, Van Horn, Finch, Smith & Caldwell (1944) who noted also an inverse relationship between the nitrogen content of the fruit and the ascorbic acid content. Several investigators, however, including Karikka, Dudgeon & Hauck (1944) and Hamner, Bernstein & Maynard (1945), who worked on potatoes and tomatoes respectively, found that the effect of nitrogenous fertilizers on the ascorbic acid content was overshadowed by the effect of environmental factors such as soil and light intensity. Moreover, as has been pointed out by Hamner *et al.* (1945), the effect of nitrogen *per se* is difficult to assess since nitrogenous fertilizers inevitably affect the size of crop, amount of foliage, etc., which have important secondary effects on the synthesis of ascorbic acid.

In the present work we have investigated the influence of nitrogenous and other salts on the synthesis of ascorbic acid, and in doing so have eliminated as far as possible the effect of other factors. We used as experimental material seedlings germinated on filter paper in dishes, since under these conditions factors such as temperature, intensity of illumination and supply of nutrients could more easily be controlled.

Giroud (1938) has pointed out that many fruits which are rich in ascorbic acid are rich also in carotene. Moreover, the work of Neubauer (1939), Moldtmann (1939) and Weber (1940) suggests that the co-existence of ascorbic acid and carotene is not accidental. In view of the possibility that the synthesis of carotene may be related to that of ascorbic acid, we also examined the effect of the different treatments on the synthesis of carotene.

METHODS

Conditions of germination. The seeds were germinated on ashless filter paper in glass crystallizing dishes, provided with glass covers to prevent evaporation. The dishes in any one experiment were subjected to identical conditions of temperature and illumination, the latter being provided by a north light. Since work was carried out at different seasons, illumination and temperature varied slightly as between individual experiments, the latter fluctuating between 15 and 20°. The seeds were kept moist during germination by the frequent addition of small amounts of the solutions being tested.

Composition of solutions used for germination. In certain of these experiments, a nutrient solution composed as follows was used:

$CaCl_2$	0·8 g.
KH_2PO_4	0·1 g.
K_2HPO_4	0·1 g.
$MgSO_4.7H_2O$	0·1 g.
$FeCl_3$	Trace

Glass-distilled water to 1 l.

The salts being tested were dissolved either in glass-distilled water (G.D. water), or in the nutrient solution in accordance with the control used, e.g. if nutrient solution was used as control the salts were dissolved in this, though for the sake of brevity, this has not been specifically indicated in the tables.

Reagents. The salts used were of A.R. purity.

Estimation of ascorbic acid. Ascorbic acid was estimated chemically with 2:6-dichlorophenolindophenol, by means of a photoelectric absorptiometer. The procedure adopted was similar to, though not identical with, that used by Bessey (1938). Reference to Table 1 shows that differences between duplicate analyses were so small as to be negligible. Dehydroascorbic acid was estimated after reduction by H_2S.

Since certain of the salts used in our experiments have to some extent affected growth as well as the synthesis of ascorbic acid, we have expressed our results not only in terms of concentration (mg./g. of fresh seedling) but also as the total amount produced by 100 seedlings, which was the number taken for each analysis. In those instances where growth was appreciably affected, the total amount synthesized gives a better indication of the effect of the nutrient supplied.

For the estimation of ascorbic acid, the complete seedling, dissected from the testa, was taken for analysis, after removal of adhering moisture.

Estimation of carotene. As with ascorbic acid, carotene was estimated in the complete seedling from which the testa and adhering moisture had been removed. Seedlings were generally taken from batches which were also used for ascorbic acid estimations. The chromatographic method of Booth (1945) was employed, with alumina-sodium sulphate as adsorbent. The precautions mentioned by him, e.g. extraction in dim light, were taken to avoid destruction of the carotene. Fifty seedlings were taken for analysis, and all analyses were done in duplicate, the figures reported in

the tables being the mean of such duplicates. The standard error of duplicates was ±0·404, which amounts to ±3·65% of the mean of the duplicates. The agreement was, therefore, satisfactory. The carotene (in light petroleum solution) was estimated by means of a photoelectric absorptiometer incorporating a Wratten filter no. 47. The instrument was calibrated with solutions of pure β-carotene. Results obtained with this colorimeter agreed well with those obtained in a Pulfrich photometer.

Estimation of pH *of cell sap.* Seedlings (2 g.) were thoroughly washed with distilled water and ground to a fine mixture with 5 ml. of distilled water in a glass mortar. The pH was determined immediately by means of the glass electrode.

RESULTS

Synthesis of ascorbic acid

Effect of ammonium sulphate on synthesis of ascorbic acid in cress seedlings. When cress was grown under the conditions described above, in the presence of $(NH_4)_2SO_4$ a considerable reduction in the concentration and total amount of ascorbic acid synthesized was observed. This occurred not only when the salt constituted the sole nutrient, but also when it was supplied in conjunction with a nutrient solution containing Ca^{++}, K^+, Mg^{++}, Fe^{+++}, Cl^-, $PO_4^{\equiv}$ and $SO_4^{=}$ (see Table 1). The effect of $(NH_4)_2SO_4$ in decreasing the production of ascorbic acid was not a temporary phenomenon, but was observed during all stages of development up to 170 hr.

The extent to which the synthesis of ascorbic acid was reduced depended on the concentration of $(NH_4)_2SO_4$ present (Table 2). Growth was affected with the highest concentration (0·1 M) tested, but with a concentration of 0·02 M there was little or no impairment of growth, though the amount of ascorbic acid formed was greatly reduced. These

Table 1. *Effect of* $(NH_4)_2SO_4$ *on the synthesis of ascorbic acid in cress seedlings*

				Ascorbic acid synthesized			
				Concentration		Total produced by 100 seedlings	
Exp. no.	Treatment	Duration of exp. (hr.)	Wt. of 100 seedlings (g.)	(mg./g.)	(% of control)	(mg.)	(% of control)
1	Control (G.D.* water)	74	1·43	0·52	—	0·74	—
	$(NH_4)_2SO_4$, 0·02 M	74	1·57	0·095	18·2	0·15	20·0
2	Control (G.D. water)	75	1·62	0·54	—	0·87	—
	$(NH_4)_2SO_4$, 0·02 M	75	1·72	0·10	18·6	0·17	19·6
3	Control (G.D. water) (1)	90	1·75	0·51 } 0·50	—	0·89 } 0·875	—
	Control (G.D. water) (2)	90	1·79	0·49 }	—	0·86 }	—
	$(NH_4)_2SO_4$, 0·02 M (1)	90	1·67	0·217 } 0·218	43·7	0·35 } 0·35	40·0
	$(NH_4)_2SO_4$, 0·02 M (2)	90	1·62	0·22 }		0·35 }	
	Control (nutrient solution) (1)	90	1·84	0·42 } 0·395	—	0·78 } 0·775	—
	Control (nutrient solution) (2)	90	2·1	0·37 }	—	0·77 }	—
	Nutrient solution + $(NH_4)_2SO_4$, 0·02 M (1)	90	1·77	0·147 } 0·14	36·0	0·26 } 0·27	35·0
	Nutrient solution + $(NH_4)_2SO_4$, 0·02 M (2)	90	1·90	0·14 }		0·28 }	
4	Control (nutrient solution)	89	2·03	0·33	—	0·67	—
	Nutrient solution + $(NH_4)_2SO_4$, 0·02 M	89	1·90	0·06	18·0	0·11	16·0

* G.D. = glass distilled.

Table 2. *Effect of concentration of* $(NH_4)_2SO_4$ *on the synthesis of ascorbic acid in cress seedlings*

			Ascorbic acid synthesized			
			Concentration		Total produced by 100 seedlings	
Treatment	Duration of exp. (hr.)	Wt. of 100 seedlings (g.)	(mg./g.)	(% of control)	(mg.)	(% of control)
Control (nutrient solution)	96	2·02	0·31	—	0·62	—
$(NH_4)_2SO_4$, 0·002 M	96	2·33	0·30	97	0·69	110
$(NH_4)_2SO_4$, 0·004 M	96	2·25	0·28	90	0·62	100
$(NH_4)_2SO_4$, 0·01 M	96	2·11	0·25	80	0·53	85
$(NH_4)_2SO_4$, 0·02 M	96	1·99	0·11	35	0·21	34
$(NH_4)_2SO_4$, 0·04 M	96	1·90	0 09	29	0·18	29
$(NH_4)_2SO_4$, 0·1 M	168*	0·76	0·12	39	0·09	15

* Germination delayed.

results are of interest in that they show that a reduction in concentration of ascorbic acid may take place, unaccompanied by any appreciable depression of growth, a finding in accord with the observations of Ray (1934) on germinating pea seedlings.

Dehydroascorbic acid was also determined in some experiments. The amount found was no greater in the seedlings grown in the presence of $(NH_4)_2SO_4$ than in those grown in control solutions. The smaller amount of ascorbic acid produced in the former was, therefore, not due to any alteration in the balance between the amounts of reduced and oxidized vitamin present (Table 3).

solution. Since several workers have shown that illumination aids the synthesis of ascorbic acid by the plant, it was thought possible that the inhibiting effect of ammonium sulphate on chlorophyll formation might be the cause of the reduction in the ascorbic acid synthesized. In this event, the depressing effect of $(NH_4)_2SO_4$ should not be observed in seedlings in which the formation of chlorophyll was prevented by the complete absence of light.

The results (Table 4) show that, although the total amount of ascorbic acid synthesized was reduced in the dark, the relative amounts synthesized in seeds

Table 3. *Effect of* $(NH_4)_2SO_4$ *on the synthesis of ascorbic acid and dehydroascorbic acid in cress seedlings*

			Ascorbic acid		Dehydroascorbic acid		Total ascorbic acid (D.H.A. + A.A.)	
Exp. no.	Treatment	Duration of exp. (hr.)	Concentration (mg./g.)	Total produced by 100 seedlings (mg.)	Concentration (mg./g.)	Total produced by 100 seedlings (mg.)	Concentration (mg./g.)	Total produced by 100 seedlings (mg.)
1	Control (G.D. water)	90	0·485	0·86	0·08	0·14	0·565	1·00
	$(NH_4)_2SO_4$, 0·02 M	90	0·215	0·35	0·085	0·13	0·30	0·48
2	Control (nutrient solution)	90	0·365	0·77	0·051	0·105	0·42	0·87
	Nutrient solution + $(NH_4)_2SO_4$, 0·02 M	90	0·145	0·28	0·06	0·11	0·20	0·39

Table 4. *The production of ascorbic acid in cress seedlings germinated in different salt solutions in the presence and absence of light*

				Ascorbic acid synthesized			
				Concentration		Total produced by 100 seedlings	
Exp. no.	Treatment	Duration of exp. (hr.)	Wt. of 100 seedlings (g.)	(mg./g.)	(% of control)	(mg.)	(% of control)
1	Control (G.D. water)	72, light	1·65	0·45	—	0·74	—
	$(NH_4)_2SO_4$, 0·02 M	72, light	1·8	0·048	11	0·086	12
	Control (G.D. water)	72, dark	2·3	0·23	—	0·54	—
	$(NH_4)_2SO_4$, 0·02 M	72, dark	2·04	0·028	12	0·057	11
2	Control (G.D. water)	76, light	—	0·52	—	—	—
	NH_4NO_3, 0·02 M	76, light	—	0·50	96	—	—
	KNO_3, 0·04 M	76, light	—	0·61	117	—	—
	$(NH_4)_2SO_4$, 0·02 M	76, light	—	0·15	29	—	—
	NH_4Cl, 0·04 M	76, light	—	0·16	31	—	—
	Control (G.D. water)	125, light	—	0·59	—	—	—
	NH_4NO_3, 0·02 M	125, light	—	0·48	82	—	—
	KNO_3, 0·04 M	125, light	—	0·66	112	—	—
	$(NH_4)_2SO_4$, 0·02 M	125, light	—	0·16	27	—	—
	NH_4Cl, 0·04 M	125, light	—	0·154	26	—	—
	Control (G.D. water)	54, dark	—	0·37	—	—	—
	NH_4NO_3, 0·02 M	54, dark	—	0·35	95	—	—
	KNO_3, 0·04 M	54, dark	—	0·39	106	—	—
	$(NH_4)_2SO_4$, 0·02 M	54, dark	—	0·18	49	—	—
	NH_4Cl, 0·04 M	54, dark	—	0·18	49	—	—

The effect of ammonium salts in the presence and absence of light. It was noticed that cress seeds germinated in the presence of $(NH_4)_2SO_4$ solutions appeared less green and contained less chlorophyll than seeds germinated in water or the nutrient

grown with and without $(NH_4)_2SO_4$ remained approximately the same as in seeds grown in the light. It is clear, therefore, that the effect of $(NH_4)_2SO_4$ is not due merely to any influence which it has on photosynthetic activity.

Table 5. *Effect of* KNO_3 *on the synthesis of ascorbic acid in cress seedlings*

Exp. no.	Treatment	Duration of exp. (hr.)	Wt. of 100 seedlings (g.)	Ascorbic acid synthesized: Concentration (mg./g.)	Concentration (% of control)	Total produced by 100 seedlings (mg.)	Total produced by 100 seedlings (% of control)
1	Control (nutrient solution)	89	2·03	0·33	—	0·67	—
	KNO_3, 0·04 M	89	1·95	0·42	127	0·815	122
2	Control (G.D. water)	68	1·42	0·39	—	0·55	—
	KNO_3, 0·04 M	68	1·31	0·39	100	0·51	93
	Control (G.D. water)	92	2·05	0·33	—	0·68	—
	KNO_3, 0·04 M	92	2·32	0·35	106	0·81	119
	Control (G.D. water)	140	2·98	0·27	—	0·79	—
	KNO_3, 0·04 M	140	3·93	0·20	74	0·80	101
3	Control (nutrient solution)	48	—	0·47	—	—	—
	KNO_3, 0·04 M	48	—	0·45	96	—	—
	Control (nutrient solution)	72	—	0·44	—	—	—
	KNO_3, 0·04 M	72	—	0·59	134	—	—
4	Control (G.D. water)	76	—	0·52	—	—	—
	KNO_3, 0·04 M	76	—	0·61	117	—	—

Table 6. *Effect of different ammonium salts on the synthesis of ascorbic acid in seedlings*

Exp. no.	Treatment	Type of seed	Duration of exp. (hr.)	Wt. of 100 seedlings (g.)	Ascorbic acid synthesized: Concentration (mg./g.)	Concentration (% of control)	Total produced by 100 seedlings (mg.)	Total produced by 100 seedlings (% of control)
1	Control (G.D. water)	Cress	68	1·42	0·39	—	0·55	—
	NH_4Cl, 0·04 M	,,	68	1·35	0·048	12·0	0·066	12·0
	NH_4NO_3, 0·02 M	,,	68	1·53	0·32	82·0	0·49	89·0
	$(NH_4)_2SO_4$, 0·02 M	,,	68	1·30	0·06	15·0	0·08	15·0
	$(NH_4)_2HPO_4$, 0·02 M	,,	68	1·17	0·15	39·0	0·17	31·0
	Control (G.D. water)	,,	92	2·05	0·33	—	0·68	—
	NH_4Cl, 0·04 M	,,	92	1·59	0·03	9·0	0·05	7·0
	NH_4NO_3, 0·04 M	,,	92	1·94	0·23	70·0	0·45	66·0
	$(NH_4)_2SO_4$, 0·02 M	,,	92	1·64	0·07	21·0	0·12	18·0
	$(NH_4)_2HPO_4$, 0·02 M	,,	92	1·82	0·17	51·0	0·31	46·0
	Control (G.D. water)	,,	140	2·98	0·27	—	0·79	—
	NH_4Cl, 0·04 M	,,	140	2·21	0·05	19·0	0·10	13·0
	NH_4NO_3, 0·04 M	,,	140	2·68	0·17	63·0	0·46	58·0
	$(NH_4)_2SO_4$, 0·02 M	,,	140	2·58	0·05	19·0	0·14	18·0
	$(NH_4)_2HPO_4$, 0·02 M	,,	140	2·81	0·13	48·0	0·37	47·0
	NH_4 acetate, 0·04 M*	,,	140	1·71	0·36	134·0	0·62	79·0
2	Control (G.D. water)	Cress	120	2·35	0·34	—	0·77	—
	NH_4HCO_3, 0·02 M†	,,	120	2·23	0·35	103·0	0·78	101·0
3	Control (G.D. water)	Wheat	100	9·5	0·34	—	3·2	—
	NH_4 acetate, 0·04 M	,,	100	6·5	0·36	106·0	2·35	74·0
	NH_4Cl, 0·04 M	,,	100	6·3	0·25	74·0	1·56	49·0
	NH_4NO_3, 0·02 M	,,	100	9·4	0·32	94·0	3·0	96·0
	$(NH_4)_2SO_4$, 0·02 M	,,	100	6·6	0·25	74·0	1·68	52·0
	Control (G.D. water)	,,	124	10·8	0·28	—	3·1	—
	NH_4 acetate, 0·04 M	,,	124	9·8	0·35	125·0	3·35	108·0
	NH_4Cl, 0·04 M	,,	124	9·6	0·24	86·0	2·29	74·0
	NH_4NO_3, 0·02 M	,,	124	12·9	0·31	111·0	4·0	128·0
	$(NH_4)_2SO_4$, 0·02 M	,,	124	8·8	0·25	90·0	2·2	71·0
	Control (G.D. water)	,,	148	13·0	0·34	—	4·4	—
	NH_4 acetate, 0·04 M	,,	148	12·5	0·38	112·0	4·7	107·0
	NH_4Cl, 0·04 M	,,	148	10·6	0·23	68·0	2·2	50·0
	NH_4NO_3, 0·02 M	,,	148	14·6	0·34	100·0	5·0	114·0
	$(NH_4)_2SO_4$, 0·02 M	,,	148	8·8	0·26	76·0	2·3	52·0

* Estimations at 68 and 92 hr. were not possible since germination was delayed.

† Solution of ammonia, 0·02 M, saturated with CO_2.

Effect of potassium nitrate. Seeds were germinated in an aqueous solution of KNO_3 (0·04 M), or in a solution of KNO_3 (0·04 M) in nutrient solution. The concentration of nitrogen in the medium, therefore, was comparable with that used in the previous experiments. KNO_3 in this concentration did not depress the synthesis of ascorbic acid. In most cases, indeed, rather more ascorbic acid was synthesized than in the controls (Table 5).

Effect of asparagine. In another experiment nitrogen was supplied as asparagine, which had no influence on the synthesis of ascorbic acid. Thus the amount synthesized by seedlings grown in an asparagine solution (0·02 M) was 94, 112 and 100 % of that formed in seedlings grown in distilled water after periods of germination of 68, 92 and 140 hr., respectively.

Effect of different cations combined with anions utilized to varying extents by the plant

Effect of various cations combined with a 'non-utilizable' anion. From the above results it appeared that NH_4^+ *per se* was not the sole operative factor, but that the extent to which the anion was utilized or disposed of by the plant was of major importance. In agreement with this hypothesis was the fact that the amount of ascorbic acid synthesized by seedlings grown in the nutrient solution (which contained most of the anions in the form of $SO_4^{=}$ or Cl^-) was always lower than the amount synthesized by seedlings grown in water. The effect of various cations when combined with a 'non-utilizable' anion, viz. chloride, was therefore further investigated. The results of experiments (Table 7) in which cress seedlings were grown in solutions of chlorides of Mg^{++}, Ca^{++}, K^+ and NH_4^+ showed that, whilst the Mg, Ca and K salts all slightly depressed the synthesis of ascorbic acid, the effect of NH_4Cl was much greater.

Table 7. *The effect of different chlorides on the synthesis of ascorbic acid in cress seedlings*

Exp. no.	Treatment	Duration of exp. (hr.)	Wt. of 100 seedlings (g.)	Ascorbic acid synthesized: Concentration (mg./g.)	Concentration (% of control)	Total produced by 100 seedlings (mg.)	Total produced by 100 seedlings (% of control)
1	Control (G.D. water)	66	1·62	0·57	—	0·92	—
	$MgCl_2$, 0·02 M	66	1·82	0·39	69	0·71	77
	NH_4Cl, 0·04 M	66	1·82	0·12	21	0·22	24
	$CaCl_2$, 0·02 M	66	1·90	0·46	81	0·88	96
	KCl, 0·04 M	66	1·91	0·46	81	0·88	96
	NaCl, 0·04 M	66	2·20	0·32	56	0·71	77
2	Control (G.D. water)	90	2·28	0·43	—	0·99	—
	$MgCl_2$, 0·02 M	90	2·72	0·27	63	0·73	74
	NH_4Cl, 0·04 M	90	2·18	0·12	28	0·26	26
	$CaCl_2$, 0·02 M	90	2·72	0·28	65	0·78	79
	KCl, 0·04 M	90	2·94	0·30	70	0·88	89
	NaCl, 0·04 M	90	2·88	0·28	65	0·81	82

The effect of different ammonium salts. Although the results recorded above suggested that nitrogen in the form of ammonium salts had an effect which differed from that of nitrogen in nitrate or asparagine, in subsequent experiments it became evident that the effect of the ammonium salts varied according to the nature of the anion (Table 6). Thus when NH_4^+ was combined with Cl^- or $SO_4^{=}$, anions which are presumably not readily utilized in metabolic processes, the depressing effect was much greater than when it was combined with anions like HCO_3^-, NO_3^- or CH_3COO^-, which can be readily disposed of or transformed into other substances by the plant. The depressing effect of $(NH_4)_2HPO_4$ was intermediate, an observation in accord with the fact that $PO_4^{\equiv}$ is more readily utilized than Cl^- or $SO_4^{=}$, but less readily than HCO_3^-, NO_3^-, etc. It is of interest to note that, although CH_3COONH_4 delayed germination considerably, the amount of ascorbic acid ultimately synthesized was high.

It seems, therefore, that when the seed is germinated in the presence of a salt containing an anion not readily utilized for metabolic purposes, the synthesis of ascorbic acid is somewhat depressed. When, in addition, such an anion is combined with NH_4^+, the depressing effect is much more pronounced. These results might be interpreted on the basis of the theory that the factor which favours the synthesis of ascorbic acid by the seedling is the amount of effective base resulting from the utilization of the anion by the plant. With ammonium salts the cation itself is utilized to a greater degree than the cations in other salts, and this still further reduces the amount of base available.

Table 8. *Comparison of the effect of different potassium or sodium salts on the synthesis of ascorbic acid in cress seedlings*

Exp. no.	Treatment	Duration of exp. (hr.)	Wt. of 100 seedlings (g.)	Ascorbic acid synthesized: Concentration (mg./g.)	Concentration (% of control)	Total produced by 100 seedlings (mg.)	Total produced by 100 seedlings (% of control)
1	Control (G.D. water)	144	2·15	0·35	—	0·74	—
	K acetate, 0·02 M	144	1·85	0·58	166	1·07	144
	$KHCO_3$, 0·02 M	144	2·35	0·45	128	1·06	143
	NH_4HCO_3, 0·02 M	144	2·02	0·38	109	0·76	102
2	Control (nutrient solution)	120	2·26	0·25	—	0·57	—
	$KHCO_3$, 0·02 M	120	2·19	0·35	140	0·77	136
	$NaHCO_3$, 0·02 M	120	2·03	0·38	152	0·77	136
	K acetate, 0·02 M	120	1·72	0·46	184	0·79	139
3	Control (G.D. water)	120	1·99	0·35	—	0·69	—
	$KHCO_3$, 0·04 M	120	1·47	0·52	149	0·76	110
	KNO_3, 0·04 M	120	2·3	0·44	126	1·0	145
	KCl, 0·04 M	120	2·28	0·24	68	0·55	80
	K_2SO_4, 0·02 M	120	1·82	0·30	86	0·55	80
4	Control (nutrient solution)	144	2·8	0·20	—	0·56	—
	$KHCO_3$, 0·02 M	144	2·7	0·27	135	0·73	130
	$KHCO_3$, 0·04 M	144	2·26	0·36	180	0·82	147

Table 9. *The effect on ascorbic acid synthesis in cress seedlings of* $(NH_4)_2SO_4$ *in the presence of succinic, malic or aspartic acids, or their sodium salts*

Exp. no.	Treatment	Duration of exp. (hr.)	Wt. of 100 seedlings (g.)	Ascorbic acid synthesized: Concentration (mg./g.)	Concentration (% of control)	Total produced by 100 seedlings (mg.)	Total produced by 100 seedlings (% of control)
1	Control (G.D. water)	75	1·62	0·54	—	0·87	—
	Succinic acid, 0·02 M	75	1·69	0·59	109	1·0	115
	Succinic acid, 0·02 M + $(NH_4)_2SO_4$, 0·02 M	75	1·88	0·12	22	0·22	25
	$(NH_4)_2SO_4$, 0·02 M	75	1·72	0·10	19	0·17	20
2	Control (G.D. water)	120	2·27	0·27	—	0·62	—
	Sodium succinate, 0·02 M	120	2·23	0·40	148	0·88	142
	Sodium succinate, 0·02 M + $(NH_4)_2SO_4$, 0·02 M	120	2·04	0·24	89	0·49	79
	$(NH_4)_2SO_4$, 0·02 M	120	1·61	0·11	40	0·18	29
3	Control (G.D. water)	120	2·3	0·375	—	0·87	—
	$(NH_4)_2SO_4$, 0·02 M	120	1·68	0·144	39	0·24	28
	Malic acid, 0·02 M + $(NH_4)_2SO_4$, 0·02 M	120	1·61	0·15	40	0·25	29
4	Control (G.D. water)	144	2·15	0·41	—	0·87	—
	$(NH_4)_2SO_4$, 0·02 M	144	1·60	0·14	34	0·23	27
	$(NH_4)_2SO_4$, 0·02 M + sodium malate, 0·02 M	144	1·46	0·33	81	0·48	55
	Sodium malate, 0·02 M	144	1·75	0·58	142	1·01	116
5	Control (G.D. water)	72	1·62	0·54	—	0·88	—
	Aspartic acid, 0·02 M	72	1·76	0·54	100	0·95	108
	Aspartic acid, 0·02 M + $(NH_4)_2SO_4$, 0·02 M	72	1·70	0·11	20	0·19	22
	$(NH_4)_2SO_4$, 0·02 M	72	1·72	0·10	19	0·17	19
	Sodium aspartate, 0·02 M	72	1·67	0·63	116	1·05	119
	Sodium aspartate, 0·02 M + $(NH_4)_2SO_4$, 0·02 M	72	1·9	0·22	41	0·42	48

Effect of cations (other than NH_4^+) combined with 'utilizable' anions. On the assumption that the above theory is correct, the supply of salts such as the bicarbonates, nitrates or acetates of Na or K to seedlings should stimulate the synthesis of ascorbic acid, for in these cases the anion can either be disposed of or transferred into other substances, thus providing a potential reserve of K^+ or Na^+ ions. A comparison of the effect of these salts and of KCl and K_2SO_4 is shown in Table 8. Solutions of $KHCO_3$, $NaHCO_3$, CH_3COOK or KNO_3 all increased the synthesis of ascorbic acid when added alone or in conjunction with the nutrient solution. On the other hand, where the base was combined with a 'non-utilizable' anion such as $SO_4^=$ or Cl^-, the effect of the salt was to reduce the amount of the vitamin synthesized.

Effect of 'utilizable' organic anions and their sodium salts. The view that the amount of base liberated as the result of metabolic change is the determining factor in the synthesis of ascorbic acid is further supported by the data recorded in Table 9. In these experiments, the effect of certain organic acids and their sodium salts in modifying the action of $(NH_4)_2SO_4$ was studied. Succinic, malic and aspartic acids were chosen because of the part they are known to play in intermediary metabolism. In so far as the succinate, malate or aspartate ion is used as an intermediate, a supply of these acids in the form of their sodium salts will serve as a potential reserve of base. It was found that (1) the free acids when supplied alone did not appreciably affect the synthesis of ascorbic acid, (2) the sodium salts caused a distinct increase, and (3) the depressing effect of $(NH_4)_2SO_4$ was not modified by the presence of the free acids, but was diminished by the sodium salts. In agreement with these findings is the fact that ammonium succinate (the cation and anion of which are both utilized) had little or no adverse effect on the synthesis of ascorbic acid (Table 12).

Synthesis of carotene

Effect of different ammonium salts. $(NH_4)_2SO_4$ and NH_4Cl greatly reduced the synthesis of carotene in germinating cress seedlings (Tables 10 and 11). The amount synthesized when these salts were supplied was on an average only 40% of that formed in controls. This effect was observed also when the salts were supplied in conjunction with the nutrient

Table 10. *Effect of different ammonium salts on the synthesis of carotene in cress seedlings*

Exp. no.	Treatment	Duration of exp. (hr.)	Wt. of 50 seedlings (g.)	Carotene synthesized: Concentration (μg./g.)	Concentration (% of control)	Total produced by 50 seedlings (μg.)	Total produced by 50 seedlings (% of control)
1	Control (G.D. water)	96	1·02	12·7	—	13·0	—
	$(NH_4)_2SO_4$, 0·02 M	96	0·76	9·6	76	7·3	56
2	Control (G.D. water)	90	0·99	11·8	—	11·0	—
	$(NH_4)_2SO_4$, 0·02 M	90	0·78	6·4	54	5·0	45
3	Control (nutrient solution)	90	1·0	12·2	—	12·2	—
	$(NH_4)_2SO_4$, 0·02 M	90	1·0	4·7	39	4·7	39
4	Control (nutrient solution)	96	1·04	14·4	—	7·5	—
	$(NH_4)_2SO_4$, 0·02 M	96	1·11	5·4	38	3·0	40
5	Control (G.D. water)	144	1·29	12·7	—	16·4	—
	NH_4HCO_3, 0·02 M	144	1·16	14·3	112	16·3	99
	$(NH_4)_2HPO_4$, 0·02 M	144	1·20	8·1	64	9·7	59

Table 11. *Effect of different chlorides on the synthesis of carotene in cress seedlings*

Exp. no.	Treatment	Duration of exp. (hr.)	Wt. of 50 seedlings (g.)	Carotene synthesized: Concentration (μg./g.)	Concentration (% of control)	Total produced by 50 seedlings (μg.)	Total produced by 50 seedlings (% of control)
1	Control (G.D. water)	144	1·12	13·0	—	14·5	—
	NH_4Cl, 0·04 M	144	0·83	5·3	41	4·4	30
	$MgCl_2$, 0·02 M	144	0·89	14·3	110	12·8	88
	$CaCl_2$, 0·02 M	144	1·1	11·8	91	11·9	82
	KCl, 0·04 M	144	1·0	12·9	92	12·9	88
	NaCl, 0·04 M	144	1·23	13·0	100	16·0	110
2	Control (G.D. water)	96	0·98	11·3	—	5·6	—
	KCl, 0·04 M	96	0·86	10·6	95	4·6	83

solution. When the ammonium salt contained an anion capable of being metabolized, e.g. HCO_3^-, NO_3^- or succinate, there was little or no depressing effect (Table 12). The effect of ammonium salts on the synthesis of carotene was, therefore, in general, similar to their effect on the synthesis of ascorbic acid, though quantitatively the effect was smaller. Thus, for example, in numerous experiments with $(NH_4)_2SO_4$ the average reduction in the total amount of carotene synthesized was 58 % (range 70–44 %) as compared with a reduction of ascorbic acid synthesized of 78 % (range 89–60 %). $(NH_4)_2HPO_4$, which depressed the synthesis of ascorbic acid to a lesser extent than the chlorides and sulphates of ammonia, had likewise a lesser effect on the synthesis of carotene.

Effect of chlorides. Experiments similar to those recorded in Table 7 were undertaken in which the effect of different chlorides was investigated. The results corresponded with those obtained with ascorbic acid, in that, whilst the carotene synthesized was greatly diminished by the ammonium salt, it was only slightly diminished by the other chlorides tested.

Effect of $(NH_4)_2SO_4$ in the presence of succinate or malate. Neither succinic nor malic acids when given in conjunction with $(NH_4)_2SO_4$ significantly affected the amount of carotene synthesized. When, however, the sodium salts of these acids were added to $(NH_4)_2SO_4$, the depressing effect of the latter was largely eliminated. The results with succinate are shown in Table 12. In contrast to the findings with ascorbic acid, no increase in the synthesis of carotene was observed when sodium succinate alone was given.

Synthesis of xanthophyll. It may be of interest to note that $(NH_4)_2SO_4$ did not decrease the synthesis of xanthophyll, either in the light or in the dark.

Correlation of pH *of expressed sap with synthesis of ascorbic acid and carotene*

If our assumption is correct that the amount of base liberated as a result of metabolic change is the factor which determines the extent of synthesis of ascorbic acid and carotene in the plant, then one would expect a correlation between the pH of the cell sap and the production of these two constituents. Determinations of pH of the expressed sap of cress seedlings grown in different salt solutions, and of the ascorbic acid and carotene synthesized, were therefore made. The results of some typical experiments are summarized in Table 12.

The data show that the amount of ascorbic acid synthesized was, in fact, correlated with the pH of

Table 12. *Correlation of* pH *of cell sap with synthesis of ascorbic acid and carotene in cress seedlings*

			Ascorbic acid synthesized				Carotene synthesized				
			Concentration		Total produced by 100 seedlings		Concentration		Total produced by 50 seedlings		pH of expressed sap
Exp. no.	Treatment	Duration of exp. (hr.)	(mg./g.)	(% of control)	(mg.)	(% of control)	(μg./g.)	(% of control)	(μg.)	(% of control)	
1	Control (G.D. water)	120 (light)	0·49	—	0·81	—	14·7	—	13·4	—	4·7
	$(NH_4)_2SO_4$, 0·02 M	120 (light)	0·18	37	0·23	29	6·5	44	4·1	31	4·2
	NH_4Cl, 0·04 M	120 (light)	0·13	27	0·19	24	6·9	47	4·8	36	4·2
	NH_4NO_3, 0·04 M	120 (light)	0·37	76	0·61	76	15·8	107	13·4	100	4·7
	$(NH_4)_2HPO_4$, 0·02 M	120 (light)	0·20	41	0·32	40	12·0	82	10	74	4·3
	NH_4 acetate, 0·04 M	120 (light)	0·48	98	0·68	84	12·9	88	8·7	65	4·8
	NH_4 succinate, 0·02 M	120 (light)	0·50	102	0·70	87	14·9	102	13·0	97	4·7
2	Control (G.D. water)	144 (light)	0·50	—	0·84	—	15·0	—	12·8	—	4·7
	NH_4NO_3, 0·02 M	144 (light)	0·39	78	0·63	75	16·9	113	13·1	103	4·8
	KNO_3, 0·04 M	144 (light)	0·59	118	0·96	114	19·9	133	14·7	115	5·2
	$(NH_4)_2HPO_4$, 0·02 M	144 (light)	0·17	34	0·29	35	9·6	64	7·5	59	4·4
3	Control (G.D. water)	120 (light)	0·37	—	0·76	—	13·3	—	9·6	—	4·8
	$(NH_4)_2SO_4$, 0·02 M	120 (light)	0·16	43	0·25	33	8·0	60	5·2	54	4·3
	Na succinate, 0·02 M	120 (light)	0·60	162	0·94	124	15·0	113	8·9	93	5·4
	$(NH_4)_2SO_4$, 0·02 M + Na succinate, 0·02 M	120 (light)	0·32	87	0·52	69	12·4	93	8·2	85	4·8
	NH_4 succinate, 0·02 M	120 (light)	0·35	95	0·68	90	13·8	103	10·5	109	4·8
	Succinic acid, 0·02 M	120 (light)	0·45	122	0·72	95	13·8	103	8·7	91	4·8
	Succinic acid, 0·02 M + $(NH_4)_2SO_4$, 0·02 M	120 (light)	0·17	46	0·23	30	6·9	52	3·9	41	4·3
4	Control (G.D. water)	100 (light)	0·37	—	0·79	—	10·3	—	11·5	—	4·8
	$NaHCO_3$, 0·02 M	100 (light)	0·46	124	0·95	120	11·8	115	11·8	103	5·25
	$KHCO_3$, 0·02 M	100 (light)	0·495	134	1·05	133	11·3	110	10·9	95	5·20
	K acetate, 0·02 M	100 (light)	0·73	197	1·21	153	13·4	130	12·1	106	5·18
	NH_4HCO_3, 0·02 M	100 (light)	0·43	116	0·82	103	11·5	112	11·4	99	4·80
5	Control (G.D. water)	96 (dark)	0·26	—	0·58	—	1·93	—	4·37	—	4·70
	$(NH_4)_2SO_4$, 0·02 M	96 (dark)	0·12	46	0·20	34	1·96	101	3·28	75	4·30
	KNO_3, 0·04 M	96 (dark)	0·30	115	0·74	128	2·31	120	5·87	134	4·95

the cell sap, high pH values being associated with increased synthesis, and low pH values with decreased synthesis of ascorbic acid. In seedlings grown in such solutions as KNO_3, $KHCO_3$, $NaHCO_3$, sodium succinate or potassium acetate, i.e. where the anion was presumably being preferentially utilized, the pH values of the sap were high. A low value was found when the seeds were grown in solutions of $(NH_4)_2SO_4$, NH_4Cl and $(NH_4)_2HPO_4$, where the cation was being preferentially utilized. In the former cases the pH of the sap was 0·3–0·5 unit higher, and in the latter cases about 0·3–0·5 unit lower than that of controls.

It will be seen also from Table 12 that when succinic acid was given in conjunction with $(NH_4)_2SO_4$, the pH of the cell sap and the production of ascorbic acid corresponded with those of seedlings grown in $(NH_4)_2SO_4$ alone. When, however, sodium succinate was supplied in conjunction with $(NH_4)_2SO_4$, the pH was raised to 4·8, a value equal to that of the control, and the production of ascorbic acid was increased. It is worth noting that the pH of the sap of seedlings grown in succinic acid alone was not lower than that of seedlings grown in distilled water, which suggests that the acid was being metabolized, or alternatively, was not absorbed by the plant.

Where the amount of ascorbic acid synthesized was only slightly affected, there was little or no detectable change in comparison with controls in the pH of the cell sap. In a few cases, as with seedlings grown in NH_4NO_3, the production of ascorbic acid was slightly reduced, but no detectable change in the pH of the sap was observed. This failure to establish a more exact correlation may have been due to the imperfections inherent in our method of obtaining the cell sap.

A similar correlation also existed between the pH of the cell sap and the amount of carotene synthesized. It was, however, less close than for ascorbic acid. Whilst a lowering of pH caused a distinct fall in the carotene synthesized in all experiments, a rise in pH slightly increased the production of carotene in some experiments, but not in all (Table 12).

Though it is evident that the production of ascorbic acid and carotene in cress is correlated with the changes in the pH of the cell sap, it is possible that some other factor may have been the primary cause. This point needs emphasis, since in preliminary experiments of a similar nature with wheat, no close correlation was found between the pH of the cell sap and the amount of ascorbic acid synthesized. The decrease in ascorbic acid production was, however, considerably less than that observed with cress. On the other hand with peas, $(NH_4)_2SO_4$ reduced the synthesis of ascorbic acid by 40–60% and this was accompanied by a reduction of 0·2–0·3 in the pH of the cell sap. The degree to which different species are affected by nutrient salts evidently varies.

SUMMARY

1. The synthesis of ascorbic acid and of carotene was decreased when cress seedlings were grown (in the presence or absence of light) in solutions of $(NH_4)_2SO_4$, NH_4Cl or $(NH_4)_2HPO_4$. A similar effect was observed when the salts were supplied in conjunction with a nutrient solution.

2. The synthesis of ascorbic acid and carotene was not affected by NH_4HCO_3, NH_4NO_3, ammonium acetate or ammonium succinate.

3. Ascorbic acid (but not carotene) was increased by the sodium and potassium salts of nitric, carbonic, acetic, succinic, malic or aspartic acids, although the free succinic, malic or aspartic acids had little or no influence.

4. The depressing effect of $(NH_4)_2SO_4$ was diminished by the addition of the sodium salts of succinic, malic or aspartic acid, but not by the free acids.

5. The synthesis of xanthophyll was not diminished in cress seedlings grown in solutions of $(NH_4)_2SO_4$.

6. There was a positive correlation between the pH value of the expressed cell sap and the amounts of ascorbic acid synthesized. A similar correlation was less close in the case of carotene.

7. The observed effect of the salts used may be explained by the hypothesis that the factor determining the extent of the synthesis of ascorbic acid is the amount of base becoming available to the plant after metabolic change.

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The Metabolism of Sulphonamides

4. THE RELATION OF THE METABOLIC FATE OF AMBAMIDE (MARFANIL) AND V 335 TO THEIR LACK OF SYSTEMIC ANTIBACTERIAL ACTIVITY

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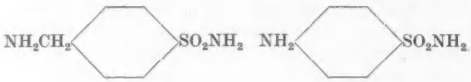
p-Aminomethylbenzenesulphonamide (I) was first described by Miller, Sprague, Kissinger & McBurney (1940), and is used in therapy as its hydrochloride, Ambamide (formerly Marfanil). Its antibacterial properties were studied by Klarer (1941) who found it to be less active than sulphanilamide against streptococci, but more active against anaerobic bacteria (cf. Schoop & Stoltz, 1941; Hamre, Walker, Dunham, van Dyke & Rake, 1944; Lawrence, 1945). It differs chemically from sulphanilamide (II) in that its amino group is aliphatic. Unlike sulphanilamide, its antibacterial action is not inhibited by *p*-aminobenzoic acid and is immediate, showing no latent

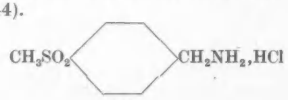


I II

period as do sulphonamides possessing an aromatic amino group (Jensen, Schmith & Brandt, 1942). Furthermore, it is not inhibited by the analogous *p*-aminomethylbenzoic acid, nor by aneurin or nicotinic acid (Schmith, 1943).

There is, however, a considerable difference between its *in vivo* and its *in vitro* activity. When given orally or intraperitoneally the drug affords no protection to mice infected with *Cl. welchii*. It gives complete protection when injected intramuscularly at the site of infection, but none if the injection is remote from the site of infection (Evans, Fuller & Walker, 1944; Siebenmann & Plummer, 1945). Ambamide has been used successfully for the treatment of surface wounds (Mitchell, Rees & Robinson, 1944) where sulphanilamide would be inhibited by pus and necrotic tissue. When used systemically the drug is of little value.

A study of the metabolism of *p*-methylsulphonylbenzylamine hydrochloride (V 335) (III) has also been included in this paper, for its antibacterial properties are similar to those of Ambamide (Evans *et al.* 1944).



III

In the present work it will be shown that the systemic inactivity of Ambamide and of V 335 is due to their rapid transformation in the body into antibacterially inactive metabolites. A clue to the mechanism of the inactivation of these drugs was provided by the observations of Beyer & Govier (1945) and of Blaschko & Duthie (1945), that Ambamide and V 335 were substrates for amine oxidase.

METHODS AND RESULTS

Experiments with Ambamide

Isolation of p-*carboxybenzenesulphonamide from Ambamide urine.* A rabbit was given, by stomach tube, 2·06 g. of Ambamide (m.p. 261°)* dissolved in water. The urine (170 ml.; alkaline to litmus) was collected during 24 hr., filtered through glass wool and centrifuged. It was treated with $\frac{1}{10}$ vol. of conc. HCl and a copious crystalline precipitate separated. After an hour at 0°, the precipitate was filtered at the pump, dried at 100° and weighed. The product (m.p. 280–285°; 2·15 g.) was recrystallized (charcoal) twice from aqueous ethanol and *p*-carboxybenzenesulphonamide was obtained as clusters of long white rods, m.p. and mixed m.p. 295–296°. (Found: C, 42·1; H, 3·55; N, 6·8; S, 15·4 %; equiv. by titration, 200·7: calc. for $C_7H_7O_4NS$: C, 41·8; H, 3·5; N, 7·0; S, 15·9 %; equiv. 201·2.) The ultra-violet absorption spectrum of *p*-carboxybenzenesulphonamide in ethanol showed maxima at $\lambda_{max.}$ 234 mμ ($\epsilon_{max.}$ 15,960), at $\lambda_{max.}$ 277 mμ ($\epsilon_{max.}$ 1474) and at $\lambda_{max.}$ 285·5 mμ ($\epsilon_{max.}$ 1228); and minima at $\lambda_{min.}$ 267 mμ ($\epsilon_{min.}$ 1105) and $\lambda_{min.}$ 283 mμ ($\epsilon_{min.}$ 1105). Synthetic *p*-carboxybenzenesulphonamide, m.p. 296°, was prepared by oxidation of *p*-toluenesulphonamide with alkaline permanganate according to Remsen & Palmer (1882).

Quantitative determination of the p-*carboxybenzenesulphonamide excreted.* Single animals were fed Ambamide by stomach tube. The urine was collected after 24 hr. and after 48 hr. It was found in all animals that the excretion of carboxybenzenesulphonamide was complete within 24 hr. Each urine was centrifuged and a sample (50–100 ml.) of the

* Melting-points are uncorrected.

clear urine was acidified with $\frac{1}{10}$ vol. of conc. HCl. The acidified urine was kept at 0° for an hour and then the precipitated sulphonamide was collected in a weighed Gooch crucible. The whole was dried at 100° and weighed. The filtrate from the precipitate was now extracted with ether for 6 hr. in a continuous extractor. The ethereal extract was evaporated and the residue dissolved in a little dilute sodium hydroxide solution, treated with charcoal and filtered. The filtrate was acidified with conc. HCl as before and the precipitate filtered and weighed.

The relative amounts of carboxybenzenesulphonamide obtained by acid precipitation and by ether extraction varied considerably. In some cases the larger amount was obtained by acid precipitation whereas in others it was obtained by ether extraction (see Table 1).

The experiment was repeated, but this time the dry residue was extracted with ethanol. The above colour reaction was performed on the ethanolic extract without positive results.

It was concluded therefore, that, in rabbits, no orally administered Ambamide in doses of 500 mg./kg. was excreted unchanged.

Attempted detection of a glycine conjugate. A possible but unlikely metabolite of Ambamide is *p*-sulphonamidohippuric acid. 18 g. of Ambamide were therefore fed to rabbits and the 24 hr. urine (640 ml.) acidified with HCl. The precipitated *p*-carboxybenzenesulphonamide was fractionated from water, but no trace of a glycine conjugate was detected.

The urinary filtrate was now brought to pH 7 with Na_2CO_3 and treated with excess saturated normal lead acetate solution. The precipitate was removed by filtration and the filtrate treated with basic lead acetate at pH 9. The precipitate was collected, washed and the lead removed by H_2S. The filtrate from the lead sulphide was taken to dryness. From the residue a little *p*-carboxybenzenesulphonamide was isolated but no trace of its glycine conjugate.

Table 1. *The output of* p*-carboxybenzenesulphonamide from rabbits after the oral administration of Ambamide*

						p-Carboxybenzenesulphonamide				
						Obtained from sample analyzed (mg.)		% of dose		
Rabbit no.	Wt. (kg.)	Dose (g.)	Dose/kg. (mg.)	Urine vol. (ml.)	Vol. of sample analyzed (ml.)	By pptn.	By extraction	By pptn.	By extraction	Total
69	2·7	2·060	768	90	50	738·2	9·3	71·4	0·9	72·3
71	2·55	2·090	820	195	100	357·2	295·9	36·8	30·6	67·4
49	2·8	1·396	499	104	98	599·0	396·2	50·4	33·2	83·6
50	3·0	1·515	505	97	92	479·8	160·0	37·0	12·3	49·3
69	2·75	1·375	500	88	84	513·8	180·0	43·3	15·2	58·5
49	2·7	1·446	535	160	145	843·8	95·2	71·4	8·0	79·4
50	2·8	1·427	510	150	145	659·9	115·1	53·0	9·2	69·2
69	2·6	1·490	573	120	115	864·5	82·9	67·0	6·4	73·4
91	2·45	0·754	308	100	80	174·2	118·1	32·0	21·7	53·7
92	2·3	0·739	321	110	100	278·4	114·2	45·9	18·8	64·7
93	2·5	0·776	310	110	100	292·5	79·0	45·9	12·4	58·3
94	2·1	0·585	280	100	90	31·2	248·8	6·6	52·4	59·0
95	1·9	0·557	293	100	90	0	181·4	0	40·1	40·1
96	1·9	0·538	283	110	100	0	194·4	0	44·0	44·0

A colour reaction for Ambamide and the attempted detection of the free drug in urine. Equimolecular proportions of free *p*-aminomethylbenzenesulphonamide (m.p. 153°, prepared from Ambamide, cf. Klarer, 1941) and 2:4-dinitrobenzaldehyde were heated for 5–10 min. in ethanolic solution containing a little glacial acetic acid. A yellow solution was obtained (probably containing a Schiff's base) which turned a deep purple when made alkaline with NaOH. The colour faded to brownish in about 10 min.

Six rabbits received collectively 6·5 g. of Ambamide orally. A volume of 500 ml. of the 24 hr. urine (1635 ml.) was taken to dryness under reduced pressure. The dry residue was extracted with 25 ml. 2N-NaOH, and the alkaline solution extracted with ether. On evaporation of the ether, the residue that remained was negligible.

Ethereal sulphate output after feeding Ambamide. The ethereal sulphate output after Ambamide, was determined in rabbits carefully maintained on a constant diet. The urine was analyzed for total and inorganic sulphate by the Folin gravimetric method, the normal output of ethereal sulphate being calculated as described in earlier papers (e.g. Williams, 1938; Dobson & Williams, 1946). The results are given in Table 2.

The accuracy of estimating a rise in ethereal sulphate by this method is about ± 5 % and therefore

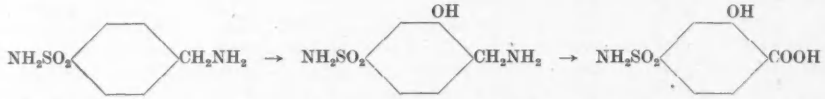
Table 2. *Ethereal sulphate output from rabbits receiving Ambamide*

Rabbit no.	Dose (mg./kg.)	Ethereal sulphate (mg. SO_3) Mean normal value	After Ambamide	Apparent rise	Ambamide equivalent (mg.)	(% of dose)
48	250	27·5	38·4	10·9	30·3	3·9
49	250	28·6	50·7	22·1	61·4	8·6
91	250	24·6	40·0	15·4	42·8	7·4
92	250	22·9	15·1	−7·8	—	0
93	250	23·6	53·2	29·6	82·3	13·2
91	500	24·2	31·9	7·7	21·4	1·8
92	500	19·7	23·2	3·5	9·7	0·9
93	500	23·5	44·7	21·2	58·9	4·6
94	500	23·0	27·8	5·8	16·1	1·4
95	500	17·5	14·2	−3·3	—	0
96	500	19·9	31·9	12·0	33·4	3·5
73	500	24·0	47·0	23·0	63·9	5·0
74	500	13·0	34·0	21·5	59·8	4·7

rises in ethereal sulphate of 5% or less are not significant. In Table 2, the figure of 5% is exceeded in three experiments at a dose level of 250 mg./kg., but never at the higher dose of 500 mg./kg. These figures, therefore, indicate a possibility but not a probability that Ambamide has undergone a nuclear oxidation. Attempts were therefore made to check this by testing Ambamide urine for possible nuclear oxidation products.

Attempts to detect nuclear oxidation products of Ambamide

If Ambamide undergoes nuclear oxidation *in vivo*, then it is likely to be oxidized in a position *ortho* to the aminomethyl group, the final product of metabolism being 3-hydroxy-4-carboxybenzenesulphonamide, thus:



Nuclear oxidation after transformation to *p*-carboxybenzenesulphonamide is unlikely, for compounds carrying two acidic groups attached to the aromatic ring are usually rapidly excreted as such. This point was checked by measurement of the ethereal sulphate output after feeding *p*-carboxybenzenesulphonamide.

(*a*) *Ethereal sulphate output after* p-*carboxybenzenesulphonamide.* This was determined as for Ambamide. The results are given in Table 3.

Table 3 shows that *p*-carboxybenzenesulphonamide does not produce an increased ethereal sulphate output. The two figures of 6·6 and 6·3 are probably within the range of experimental error of the analytical method.

(*b*) *The detection of* 3-*hydroxy*-4-*carboxybenzenesulphonamide in urine.* 3-Hydroxy-4-carboxybenzenesulphonamide, m.p. 231°, was prepared essentially according to Walker (1897) (cf. also Bromwell, 1897). (The ultra-violet absorption spectrum of this compound in ethanol showed maxima at 237·5 mμ ($\epsilon_{max.}$ 8720) and 303·5 mμ ($\epsilon_{max.}$ 3074) and minima at 226 mμ ($\epsilon_{max.}$ 6365) and 262 mμ ($\epsilon_{max.}$ 240)). This compound gives a very intense red colour with 2% aqueous ferric chloride and can be detected in small amounts in urine by this colour test. The compound was fed orally to rabbits in doses which could be expected to occur in the urine of rabbits receiving Ambamide, if the latter were converted to it to the extent of 5% at a dose level of 350 mg./kg. Two rabbits were, therefore, fed 47·5 and 67 mg. respectively of hydroxycarboxy-

Table 3. *Ethereal sulphate output from the rabbit after feeding* p-*carboxybenzenesulphonamide*

Rabbit no.	Dose (mg./kg.)	Ethereal sulphate output (mg. SO_3) Mean normal value	After the sulphonamide	Apparent rise	*p*-Carboxybenzenesulphonamide equivalent to rise (mg.)	(% of dose)
74	250	23·5	18·0	−5·5	—	0
94	250	20·9	35·1	14·2	35·5	6·6
95	250	16·9	12·7	−4·2	—	0
96	250	22·3	34·1	11·8	29·5	6·3
73	500	41·5	35·1	−6·4	—	0
75	500	25·4	25·6	0·2	—	0

benzenesulphonamide. Three hours later the urine of each rabbit was collected. Samples (2 ml.) of the urines were acidified with a few drops of conc. HCl and then shaken up with ether. The ether layer was poured into an equal volume of water. The addition of a few drops of 2% ferric chloride produced an intense red colour in the aqueous layer, which indicates that hydroxycarboxybenzenesulphonamide had been rapidly excreted in the free state.

(*c*) *Attempted detection of 3-hydroxy-4-carboxybenzenesulphonamide in Ambamide urine.* A total of 14 g. of Ambamide was administered orally to seven rabbits (dose *c.* 800 mg./kg.). The urine (800 ml.) collected during 24 hr. was pooled and clarified by centrifuging. A quantity of 760 ml. was acidified with $\frac{1}{10}$ vol. of conc. HCl, and the precipitate gave no ferric chloride reaction when dissolved in water. The filtrate was now refluxed for 2 hr. to hydrolyze conjugated phenols. The mixture was cooled and extracted with ether for 12 hr. in a continuous extractor. Evaporation of the ether yielded a residue (2·5 g.) which gave no colour test for 3-hydroxy-4-carboxybenzenesulphonamide, but yielded on recrystallization 2·2 g. of *p*-carboxybenzenesulphonamide, m.p. 294°.

Attempted detection of acetylation of Ambamide in vivo

(*a*) *Preparation of* p-*acetamidomethylbenzenesulphonamide.* Ambamide (5 g.) was dissolved in 20 ml. water and 8 g. of Na_2CO_3 in 10 ml. warm water added. To the mixture 5 ml. acetic anhydride were added, the whole shaken for about 5 min. and then kept at room temperature for 2 hr. The mixture was acidified with dilute HCl and the acetyl compound separated as white needles. Recrystallization from hot water gave 4 g., m.p. 177° (Klarer (1941) gives m.p. 177°).

(*b*) *Fate of the acetyl compound in the rabbit.* The acetyl compound (1·65 g.) was fed orally to a rabbit (dose 666 mg./kg.) and the urine collected for 24 hr. The urine was reduced to 30 ml. under diminished pressure and acidified with HCl. A crystalline precipitate (0·25 g.) separated. This was recrystallized twice (charcoal) from hot water and identified as *p*-acetamidomethylbenzenesulphonamide, m.p. and mixed m.p. 177°. No trace of *p*-carboxybenzenesulphonamide was detected by acid precipitation or ether extraction. No phenolic compound was detected in an ether extract of the hydrolyzed urine.

(*c*) *The absence of* p-*acetamidomethylbenzenesulphonamide from Ambamide urine.* In no case was an acetyl compound detected in Ambamide urine in the precipitate obtained by acidification or in the ethereal extract of the acidified urine.

Glucuronide formation. No quantitative estimations of the glucuronic acid output after feeding Ambamide were carried out, for qualitative tests indicated that no significant increase in glucuronic acid occurred.

Experiments with V 335

Isolation of p-*methylsulphonylbenzoic acid from V 335 urine.* Two rabbits were each given by stomach tube 1·5 g. V 335 (m.p. 265°) in water. The urine (240 ml.) was collected after 24 hr. and centrifuged. Conc. HCl (24 ml.) was added to the clear urine and a crystalline precipitate appeared immediately. After the acidified urine had stood a short while, the pale buff precipitate was filtered off and dried (m.p. 262°). It was twice recrystallized (charcoal) from aqueous ethanol, forming a mixture of white rods and plates; m.p. and mixed m.p. with synthetic *p*-methylsulphonylbenzoic acid (see below), 267°. Found: C, 47·9; H, 4·3; S, 16·1. $C_8H_8O_4S$ requires C, 48·0; H, 4·0; S, 16%.

Synthesis of p-*methylsulphonylbenzoic acid.* *p*-Toluenesulphonyl chloride (British Drug Houses Ltd.) was reduced to *p*-toluenesulphinic acid (m.p. 84°) with zinc dust in ether and water (Ullmann & Pasdermadjian, 1901). The acid was methylated with dimethyl sulphate and KOH yielding *p*-methylsulphonyltoluene (m.p. 85°) (cf. Otto, 1885).

p-Methylsulphonyltoluene (0·8 g.) was refluxed for 2 hr. with 3 g. $KMnO_4$ in 100 ml. water. The excess $KMnO_4$ was destroyed with ethanol and the solution filtered from the precipitate of oxides of manganese.

Table 4. *The output of* p-*methylsulphonylbenzoic acid by rabbits after oral administration of V 335*

					p-Methylsulphonylbenzoic acid				
					In sample (mg.)		% of dose		
Rabbit no.	Wt. (kg.)	Dose (mg./kg.)	Urine vol. (ml.)	Sample analyzed vol. (ml.)	By pptn.	By extraction	By pptn.	By extraction	Total
94	2·3	643	120	100	985·8	—	88·6	—	—
95	2·15	692	118	100	949·6	—	82·8	—	—
93	2·1	546	100	50	387·4	36·6	74·6	7·1	81·7
95	1·9	558	255	100	243·9	62·6	65·1	16·7	81·8
96	2·05	543	130	100	609·3	48·5	78·6	6·2	84·8
38	2·55	452	120	100	697·2	—	80·5	—	—
49	3·0	400	176	150	721·3	—	78·1	—	—

Average recovery 82·6%

The filtrate was evaporated to 50 ml. and acidified with a little HCl. The precipitated *p-methylsulphonylbenzoic acid* was recrystallized from hot water, forming a mixture of rods and plates, m.p. 267° (yield 0·4 g.). (Found: C, 47·8; H, 4·1; S, 16·1 %.)

The same acid can be obtained in poor yield by permanganate oxidation of V 335 itself.

Quantitative determination of p-*methylsulphonylbenzoic acid in V 335 urine.* The determination of this acid was carried out in exactly the same manner as described for *p*-carboxybenzenesulphonamide in Ambamide urine. This acid is much less soluble than *p*-carboxybenzenesulphonamide and therefore its determination in most cases could be carried out by acid precipitation and weighing. The results are given in Table 4.

Ethereal sulphate output after V 335. The determinations were carried out as for Ambamide. The results are given in Table 5, which shows that, within the limits of experimental error, V 335 does not cause an increased output of ethereal sulphate.

Table 5. *Ethereal sulphate output from rabbits after V 335*

Rabbit no.	Wt. (kg.)	Dose (mg./kg.)	Ethereal sulphate output (mg. SO_3) Mean normal value	After V335	Apparent rise	Rise of ethereal sulphate (% of dose)
91	2·3	504	23·0	33·2	10·2	2·0
92	2·2	495	25·1	8·0	−17·1	0
93	2·3	503	22·7	26·3	3·6	0·7

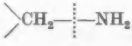
Antibacterial properties of metabolites

Through Dr A. Spinks (Imperial Chemical (Pharmaceuticals) Ltd.) both *p*-carboxybenzenesulphonamide and *p*-methylsulphonylbenzoic acid were tested for antibacterial activity. The first compound had no action on *Staph. aureus, Strep. pyogenes* or *Cl. welchii* at a dilution of 1/1000. The second compound was tested against *Staph. aureus* and *Strep. pyogenes* and found to have no activity at a dilution of 1/1000.

DISCUSSION

The present work shows that, in the rabbit, Ambamide and V 335 are rapidly transformed into metabolites which possess no antibacterial action. These results, therefore, provide an explanation of the lack of systemic activity shown by these drugs.

Evans *et al.* (1944) showed that there was a reduction in the *in vitro* activity of Ambamide and V 335, when these drugs were tested against bacteria in a medium containing whole blood. The reduction in activity in blood was ascribed 'to breakdown at the sensitive benzylamine group,



probably by an amine oxidase type of oxidation'. This view was supported by the experiments of Beyer & Govier (1945) with guinea pig liver homogenates and of Blaschko & Duthie (1945) with a preparation of amine oxidase from rabbit liver, for it was shown that both Ambamide and V 335 were substrates for amine oxidase.

The present view concerning the mechanism of the action of amine oxidase is that the primary amine is dehydrogenated to an imino compound which reacts spontaneously with water to give ammonia and an aldehyde (Richter, 1937; Pugh & Quastel, 1937). The aldehyde would then be oxidized by an aldehyde oxidizing enzyme to the corresponding acid. For Ambamide the sequence of reactions to *p*-carboxybenzenesulphonamide would be as follows:

$$NH_2.SO_2.C_6H_4.CH_2.NH_2 \rightarrow NH_2.SO_2.C_6H_4.CH{:}NH \rightarrow NH_2.SO_2.C_6H_4.CHO \rightarrow NH_2.SO_2.C_6H_4.COOH.$$

The only stage detectable in the urine is the final stage, for we have failed to get any positive tests for an aldehyde in Ambamide urine. Other work in progress in this laboratory on *p*-hydroxybenzylamine has enabled us to detect the aldehyde stage in the urine.

Our results and those of earlier workers indicate that if Ambamide or V 335 encounter amine oxidase anywhere in the body they will in all probability be dehydrogenated and inactivated. This enzyme occurs principally in the liver and intestines although it also occurs in small amounts in other tissues (Blaschko, Richter & Schlossmann, 1937; Epps, 1945). It would appear therefore that, when given by the oral route, these drugs would be oxidized during absorption from the gut and any escaping oxidation in the intestinal wall would be oxidized in the liver. Little of the unchanged drug would reach the general circulation. It has been suggested (Richter, 1938; Richter, Lee & Hill, 1941) that the function of the amine oxidase of the intestine is one of detoxication whereby toxic amines formed in the gut by bacterial action are destroyed. The lack of systemic activity of Ambamide and V 335 is therefore a consequence of this detoxicating function of amine oxidase. If derivatives of these drugs could be prepared in which the amino group is protected by a grouping which is gradually hydrolyzed *in vivo*

to the free drugs, then the antibacterial activity of these drugs might be preserved *in vivo*. With this view in mind, *p*-acetamidomethylbenzenesulphonamide was fed but was found to be largely excreted unchanged.

That Ambamide and V 335 might undergo other changes than oxidation by amine oxidase was also investigated. Although a careful search was made for metabolites other than the carboxylic acids, *p*-carboxybenzenesulphonamide and *p*-methylsulphonylbenzoic acid, none was found. An average of 70 % (highest 84 %) of the Ambamide fed was recovered as *p*-carboxybenzenesulphonamide when the dose was 500–800 mg./kg. but with the lower dose of 300 mg./kg. the average recovery was only 53 %. This difference may be due to incomplete recovery of *p*-carboxybenzenesulphonamide, when it occurs in urine at low concentrations. *p*-Carboxybenzenesulphonamide has previously been isolated from urine by Flaschenträger, Bernhard, Loewenberg & Schläpfer (1934) who found it to be a metabolite of *p*-toluenesulphonamide in the dog. With V 335 an average of 83 % (highest 87 %) of the dose (400–700 mg./kg.) was recovered as *p*-methylsulphonylbenzoic acid which appears to be a new compound although its *meta* and *ortho* isomers have been described (Smiles & Stewart, 1921; Arndt, Kirsch & Nachtwey, 1926). *p*-Methylsulphonylbenzoic acid is less soluble than *p*-carboxybenzenesulphonamide.

Concerning the mode of action of drugs of this type there is little known. Blaschko & Duthie (1945) have suggested that the drugs may interfere with enzymic systems which are in some way chemically related to amine oxidase. Evans *et al.* (1944) have pointed out that they are not inhibited by *p*-aminobenzoic acid and their chemical structures do not permit of reference to any known growth factor for bacteria. However, recently Rogers & Knight (1946) have found that in protein-free horse muscle extract there is a labile substance (toxigenic factor) which enhances α-toxin (lecithinase) formation by *Cl. welchii* type A (strain S 107). This toxigenic factor has been partially purified and shown to be of relatively small molecular size. Its toxigenic activity appears to depend on the presence of primary amino groups and it may be of the nature of an amino-sugar. We, therefore, make the suggestion that, since Ambamide and V 335 both contain aliphatic primary amino groups and are particularly active against clostridia, these drugs act by interfering with the utilization by the bacteria of substances containing aliphatic primary amino groups such as the toxigenic factor of Rogers & Knight.

SUMMARY

1. The metabolic fate of *p*-aminomethylbenzenesulphonamide hydrochloride (Ambamide) and of *p*-methylsulphonylbenzylamine hydrochloride (V 335) in the rabbit has been studied. Ambamide is rapidly oxidized and excreted in the urine as *p*-carboxybenzenesulphonamide, which is the only metabolite found. Up to 84 % of the Ambamide could be accounted for as this metabolite.

2. Ambamide does not result in the excretion of an ethereal sulphate, a glycine conjugate or a glucuronide. It does not undergo nuclear oxidation or acetylation.

3. *p*-Carboxybenzenesulphonamide and 3-hydroxy-4-carboxybenzenesulphonamide when fed to rabbits are excreted unchanged.

4. *p*-Acetamidomethylbenzenesulphonamide is excreted unchanged.

5. V 335 is excreted as *p*-methylsulphonylbenzoic acid and no other metabolite is found. Up to 87 % of V 335 can be recovered from the urine as this acid.

6. Neither *p*-carboxybenzenesulphonamide nor *p*-methylsulphonylbenzoic acid have any antibacterial activity.

7. The mechanism of the inactivation of Ambamide and V 335 is discussed.

8. A suggestion is made regarding the mode of antibacterial action of Ambamide and V 335.

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The Fate of Certain Organic Acids and Amides in the Rabbit

2. *p*-HYDROXYBENZOIC ACID AND ITS AMIDE

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Continuing our investigation of the fate of the carboxylic acid amide grouping in the rabbit we have studied the metabolism of *p*-hydroxybenzoic acid and its amide. There are several metabolic changes, involving either the hydroxyl or carboxyl group, which these compounds might undergo. In the case of the amide the carboxyl group could be formed by hydrolysis, cf. benzamide (Bray, Neale & Thorpe, 1946*a*). The hydroxyl group might be conjugated with either glucuronic or sulphuric acid and the carboxyl group with glucuronic acid or with glycine. It is also possible that a second hydroxyl group may be introduced, as with salicylic acid, which Lutwak-Mann (1943) found to give rise in the rat to gentisic acid (2:5-dihydroxybenzoic acid), and with *p*-hydroxybenzenesulphonamide, 4–5% of which was shown in this laboratory to be converted to 1:2-dihydroxybenzene-4-sulphonamide in the rabbit (Williams, 1941).

The metabolism of *p*-hydroxybenzoic acid has been studied by several workers, since it is a possible product of the putrefactive action of bacteria in the large intestine on tyrosine. Baumann & Herter (1877) showed that it caused an increase in ethereal sulphate excretion when administered to dogs. Schotten (1882) found that it gave rise to *p*-hydroxyhippuric acid in man, 16% of a 26 g. dose being excreted in this form and 35% unchanged. Quick (1932*a*, *b*) also studied this aspect of its metabolism and isolated from the urine of dogs receiving the acid a diglucuronide of *p*-hydroxybenzoic acid, both available groups being conjugated. Sherwin (1918) and Power & Sherwin (1927), however, found that in both man and the monkey the acid was excreted largely unchanged. The metabolism of the amide does not appear to have been investigated except in so far as it was included in a study of the sulphate conjugation of various derivatives of phenol by Williams (1938).

In this investigation we have examined quantitatively the excretion of free *p*-hydroxybenzoic acid, and its conjugates with glycine, sulphuric acid and glucuronic acid, the latter as ester or ether glucuronides. The amide has been similarly studied in order to estimate the extent to which the amide group can be hydrolyzed *in vivo*. We have shown that it is possible to obtain extracts of rabbit liver which are capable of hydrolyzing some aromatic amides, and the effect of nuclear substituents on this reaction is being studied. This work will be reported separately, but reference will be made in this paper to some relevant experiments. Various qualitative aspects of the metabolism of the acid and amide have also been investigated, including the isolation of protocatechuic acid and 4-carbamylphenylglucuronide from the urine of rabbits dosed with the amide. Protocatechuic acid is also present, though in smaller amounts, in the urine of rabbits receiving *p*-hydroxybenzoic acid.

METHODS

Diet and feeding. These were as described in an earlier paper (Bray *et al.* 1946*a*). The rabbits used for some of the qualitative experiments received a diet of rabbit pellets 70 g. per diem (diet no. 18, Bruce & Parkes, 1946) with water *ad libitum*. The acid was given by stomach tube as a solution of its sodium salt and the amide similarly, as a suspension in water. Five dose levels were used, viz.

0·1, 0·25, 0·5, 1·0 and 1·5 g./kg. In no case was any toxic effect observed.

Preparation of p*-hydroxybenzamide.* Methyl *p*-hydroxybenzoate (10 g.) was heated with ammonia (10 ml. S.G. 0·88) in a sealed tube at 130–140° for 8–10 hr. (cf. Hartmann, 1877). The product, a brown syrup which readily crystallized, was recrystallized from water and gave a monohydrate melting indefinitely at 90–110°, depending on the rate of heating. Above its melting-point this compound lost water, forming the anhydrous amide which melted at 161–162°. The average yield was 50% of the ester used. Considerable decarboxylation occurred during the preparation, phenol and ammonium carbonate being formed.

Preparation of p*-hydroxyhippuric acid.* This compound was prepared by the method of Fischer (1908) from glycine ester and *p*-methylcarbonatobenzoyl chloride. The product crystallized from ethanol-ether, melted at 240° after sintering at 236°. Attempts to prepare this compound by diazotization of *p*-aminohippuric acid and subsequent decomposition of the diazonium compound were unsuccessful.

Estimation of p*-hydroxybenzoic and* p*-hydroxyhippuric acids.* The method used was essentially that described by Quick (1932*a*) for substituted benzoic acids, in which the ether-soluble material from urine is fractionated by means of toluene into the unconjugated acid, which in general is soluble in toluene, and the glycine conjugate, which is insoluble. In a later paper (Quick, 1932*b*) it is stated, however, that this procedure is not applicable to *p*-hydroxybenzoic acid unless the amounts to be estimated are very small, as the acid is not readily soluble in toluene, and a method is recommended involving separate estimation of the total phenol and of glycine. This was too lengthy for our purpose, but we found that we could adapt Quick's earlier method so that quantitative separation of the ether-soluble acid by toluene could be achieved. Control experiments, in which mixtures of known amounts of the two acids were extracted from water or normal rabbit urine, gave, in 16 estimations, average recoveries corresponding to 98% of the total acid, 95% of the *p*-hydroxybenzoic acid and 101% of the *p*-hydroxyhippuric acid.

Urine (20 ml.), acidified with 2 N-H_2SO_4 (2 ml.), was continuously extracted with ether for 6 hr. The ether extract was then evaporated to dryness *in vacuo*, it being essential to dry the residue completely before extracting with toluene. This process was carried out by refluxing the residue with successive amounts of toluene (20 ml. for 45 min., followed by three consecutive volumes of 10 ml. for 20 min., decanting each time). The completeness of the extraction was checked by separate evaporation and titration of the last extract. The three extracts were combined and evaporated to dryness *in vacuo* below 50°. The solid residues (toluene-soluble and toluene-insoluble) were separately dissolved in water (20 ml.) and titrated with 0·02 N-NaOH to pH 7·8, using phenol red as indicator. It was found advisable to modify the method slightly when estimating the acids in urines passed during the first 24 hr. after a large dose of *p*-hydroxybenzoic acid. The daily volume was diluted to 450–500 ml. and only 10 ml. used for the estimation. Control experiments showed that the presence of *p*-hydroxybenzamide does not affect the estimation of ether-soluble acids by this method.

The estimation of ethereal sulphate and reducing substances (i.e. ester and ether type glucuronides) was carried out as described previously (Bray *et al.* 1946*a*, *b*). In the last estimation, control experiments showed that 90 min. hydrolysis was sufficient for complete liberation of the glucuronic acid present as ether type glucuronide.

RESULTS

Quantitative studies

Ether-soluble acid excretion. The average 'baseline' values ranged as before from 287 to 370 mg. (Bray *et al.* 1946*a*). Extraction of the ether-soluble acid with toluene as described above removed 74–80% of the total. This was greater than when light petroleum was used for the extraction (Bray *et al.* 1946*a*), when only 10–25% was removed. The nature of the normal ether-soluble acid of rabbit urine is unknown.

Table 1 shows the percentages of the doses excreted as ether-soluble acid, soluble in toluene (calculated as *p*-hydroxybenzoic acid) and as ether-soluble acid insoluble in toluene (calculated as *p*-hydroxyhippuric acid). In the case of the amide some of the results represent the total (i.e. unfractionated) acid. In every case the excretion of ether-soluble acid in the second 24 hr. after the dose was normal.

Ethereal sulphate excretion. The normal range of the daily output of ethereal sulphate of the rabbits used in this investigation was 10–38 mg. SO_3, average values for individual rabbits ranging from 16 to 27 mg. Table 2 shows the percentages of doses of acid and amide excreted conjugated with ethereal sulphate, assuming that conjugation of these compounds was the sole cause of the increase of ethereal sulphate excretion observed. The increase in ethereal sulphate excretion did not persist after 24 hr. from the time of dosing.

Excretion of reducing substances. The average normal daily output ranged from 139 to 229 mg. (calculated as glucuronic acid). These values were found to increase on acid hydrolysis as previously described, but it is now evident that the actual percentage increase may vary considerably, average values in the experiments described here corresponding to 39% of the unhydrolyzed value (range 35–40%). Table 3 shows the percentage of the doses excreted conjugated with glucuronic acid as ester and ether type glucuronides, assuming the increase in reducing values observed to be due entirely to the excretion of these compounds. The amount of reducing material excreted returned to within the normal range during the second 24 hr. after dosing.

Enzyme studies. We have been able to show that extracts of rabbit liver, the preparation of which will be described in a separate paper, are capable of hydrolyzing amides. The extent of hydrolysis can be estimated by a formol titration based upon that

described by Northrop (1932) and Balls & Lineweaver (1939). Benzamide is hydrolyzed to the extent of 50–60% of a 0·02 M solution in 48 hr. and *p*-nitrobenzamide to the extent of 50–70% of a 0·02 M solution in 5 hr. In three out of four experiments with *p*-hydroxybenzamide, carried out under exactly similar conditions, no detectable splitting occurred, while in the fourth 15% was hydrolyzed in 48 hr. These studies are being continued with a view to determining the effect of substituents and their position in the benzenoid ring, on the stability of amides to enzymic hydrolysis.

Table 1. *Excretion of ether-soluble acid (calculated as* p-*hydroxybenzoic and* p-*hydroxyhippuric acids) after doses of* p-*hydroxybenzoic acid and* p-*hydroxybenzamide*

Exp. no.	Rabbit	Approx. dose level (g./kg.)	Percentage of dose excreted as: *p*-Hydroxybenzoic acid	*p*-Hydroxyhippuric acid	Total ether-soluble acid
			p-Hydroxybenzoic acid		
27	102	0·1	28	29	57
23	118	0·1	54	18	72
14	119	0·1	21	34	55
24	119	0·1	25	29	54
13	120	0·1	37	43	80
15	102	0·25	40	19	59
26	102	0·25	47	16	63
20	118	0·25	41	30	71
30	118	0·25	54	13	67
9	119	0·25	49	21	70
17	120	0·25	40	23	63
8	102	0·5	64	16	80
7	118	0·5	74	17	91
16	118	0·5	65	20	85
29	119	0·5	72	17	89
11	120	0·5	34	6	40
21	120	0·5	56	12	68
22	120	0·5	70	6	76
10	102	1·0	49	15	64
5	118	1·0	57	16	71
6	119	1·0	45	33	78
18	119	1·0	32	26	58
12	120	1·0	68	17	85
19	102	1·5	42	20	62
25	102	1·5	47	17	64
24	119	1·5	35	24	59
22	120	1·5	53	17	70
			p-Hydroxybenzamide		
45	102	0·1	2·3	1·2	3·5
36	118	0·1	^{x}50	Nil	^{x}50
43	119	0·1	—	—	^{x}48
37	120	0·1	Nil	Nil	Nil
32	102	0·25	Nil	Nil	Nil
42	102	0·25	—	—	^{x}22
49	119	0·25	Nil	Nil	Nil
44	102	0·5	—	—	Nil
48	118	0·5	12	Nil	12
46	119	0·5	2·8	4·6	7·4
33	120	0·5	7·3	Nil	7·3

Table 1 (*cont.*).

Exp. no.	Rabbit	Approx. dose level (g./kg.)	Percentage of dose excreted as: *p*-Hydroxybenzoic acid	*p*-Hydroxyhippuric acid	Total ether-soluble acid
35	102	1·0	1·0	1·8	2·8
45	102	1·0	—	—	Nil
50	102	1·0	2·3	1·2	3·5
34	119	1·0	3·1	1·6	4·7
47	120	1·0	—	—	4·9
39	102	1·5	2·0	1·8	3·8
38	119	1·5	2·5	1·0	3·5
51	119	1·5	—	—	5·9
52	119	1·5	—	—	2·7
44	120	1·5	—	—	3·7

(1) Experiment numbers in Tables 1, 2 and 3 enable results obtained from the same experiment to be correlated.

(2) The results marked x at the lower dose levels are considered to be abnormally high and are omitted from the averages in Table 4. In these cases the actual amounts of the various metabolites excreted were usually within the normal range, although actually above the base-line used. Even such small increases represent a large percentage of doses of 200–500 mg.

(3) The conjugation of any protocatechuic acid formed is ignored since the amounts excreted are very small.

Table 2. *Percentage of doses of* p-*hydroxybenzoic acid and* p-*hydroxybenzamide excreted conjugated with ethereal sulphate*

Exp. no.	Rabbit	Approx. dose level (g./kg.)	Percentage excreted as ethereal sulphate
		p-Hydroxybenzoic acid	
1	102	0·5	7·1
4	102	0·5	6·2
10	102	1·0	6·1
25	102	1·5	4·4
30	118	0·25	6·2
3	119	0·1	3·7
2	119	0·25	8·3
24	119	1·5	7·4
12	120	1·0	4·5
53	120	1·5	3·7
		p-Hydroxybenzamide	
42	102	0·25	21·9
41	102	0·5	19·1
35	102	1·0	17·1
39	102	1·5	13·9
32	118	0·5	15·0
40	118	1·0	16·8
43	119	0·1	26·3
31	119	0·25	23·6
38	119	1·5	13·7
37	120	0·1	43·1

See footnote to Table 1.

Table 3. *Percentage of doses of* p-*hydroxybenzoic acid and its amide excreted as ether and ester type glucuronides*

Exp. no.	Rabbit	Approx. dose level (g./kg.)	Percentage of dose excreted as: Ester glucuronide	Ether glucuronide
		p-Hydroxybenzoic acid		
1	102	0·5	3·5	7·8
4	102	0·5	15·1	Nil
10	102	1·0	6·1	13·3
25	102	1·5	6·6	14·1
3	119	0·1	16·3	18·6
2	119	0·25	5·0	9·5
24	119	1·5	11·0	Nil
22	120	0·5	5·0	4·7
12	120	1·0	5·2	11·0
53	120	1·5	1·8	10·5
		p-Hydroxybenzamide		
42	102	0·25	Nil	15·1
41	102	0·5	Nil	15·4
35	102	1·0	Nil	18·0
39	102	1·5	Nil	20·2
36	118	0·1	Nil	Nil
40	118	1·5	2·8	23·8
43	119	0·1	Nil	Nil
31	119	0·25	Nil	15·6
34	119	1·0	2·5	29·3
37	120	0·1	Nil	[z]45·1

See footnote to Table 1.

Qualitative studies

Isolation of p-*hydroxybenzoic and* p-*hydroxyhippuric acids*

The urine passed by rabbits during 24 hr. after they had received *p*-hydroxybenzoic acid (1 g./kg.) was acidified (approx. 10 ml. 2N-H_2SO_4/100 ml. urine) and continuously extracted with ether for 24 hr. The extract was evaporated to dryness and extracted repeatedly with boiling toluene. The two fractions were purified as follows.

Toluene soluble fraction. The solvent was removed *in vacuo* and the residual syrup dissolved in water and treated with charcoal. The colourless filtrate deposited rosettes of white needles which melted at 207–209°. After two recrystallizations from water the melting-point was 214° alone or in admixture with an authentic sample of *p*-hydroxybenzoic acid (m.p. 214°).

Residue after toluene extraction. The brown residue was dissolved in water and decolorized by treatment with charcoal. The crystals obtained by evaporation of the filtrate melted at 215–216° and still contained an appreciable amount of *p*-hydroxybenzoic acid. This was removed by further extraction with toluene. The residue from this extraction, after recrystallization from water, melted at 239–240° with decomposition. A mixed melting-point with an authentic specimen of *p*-hydroxyhippuric acid (m.p. 240°) was 238–239°.

Isolation of 3:4-*dihydroxybenzoic acid* (*protocatechuic acid*)

This oxidation product was isolated from urine passed by rabbits which had received *p*-hydroxybenzamide. It was first detected in the urine of rabbits receiving the acid, being associated with the *p*-hydroxyhippuric acid fraction, but although this gave the characteristic bright green colour reaction with ferric chloride, crystalline specimens were only obtained from urine obtained after dosing with the amide. Such urine often gave a green colour with ferric chloride as collected. We were unable to separate protocatechuic acid from the other metabolites with which it was associated by fractional crystallization, but we were able to isolate it *via* its insoluble lead salt.

Urine from 10 rabbits which had been dosed with *p*-hydroxybenzamide (1·5 g./kg.) was acidified (2 ml. 2N-H_2SO_4/100 ml.) and continuously extracted with ether for 48 hr. The extract was evaporated to dryness and gave, in aqueous solution, a greenish coloration with ferric chloride. It was, however, shown that almost all the ether-soluble material was *p*-hydroxybenzamide which had been excreted unchanged. The small amount of protocatechuic acid present could not be isolated.

The urine after ether extraction was hydrolyzed by boiling for 20 min. with 0·2 vol. conc. HCl, neutralized to congo red and continuously extracted with ether for 60 hr. The ether-soluble material, after removal of the solvent, was dissolved in water and decolorized with charcoal. On concentration of the filtrate a large crop of crystals was obtained. These were shown to consist of *p*-hydroxybenzoic acid, derived from the amide by the acid hydrolysis. The mother liquors, which gave an intense green colour with ferric chloride, were treated with lead acetate solution (0·5N) until no further precipitation occurred. The precipitate was centrifuged off, suspended in water and decomposed with H_2S. The filtrate from lead sulphide was evaporated to dryness and the residue repeatedly extracted with absolute ethanol. The combined extracts were evaporated to dryness and the resulting syrup dissolved in water. By gentle evaporation of this solution fine white needles were obtained, melting at 191°. Recrystallization from water raised the melting-point to 195–196°. A mixed melting-point with an authentic specimen of protocatechuic acid (m.p. 199°) was 198°; yield 100 mg. from 30 g. amide fed. The identity of the product was further confirmed by its conversion to the diacetyl derivative by refluxing for 15 min. with acetic anhydride. The excess anhydride was decomposed with water, the solution

concentrated and kept at 0° overnight. White crystals separated which melted at 157°. A mixed melting-point with an authentic specimen of diacetylprotocatechuic acid (m.p. 157–158°) was 155–157°.

The fact that protocatechuic acid could be isolated only after the ether-extracted urine had been hydrolyzed, suggests that it is excreted mainly in conjugated form, probably as the ethereal sulphate or ether-glucuronide of its amide, or, if the rabbit is able to hydrolyze protocatechuic acid amide, as an ethereal sulphate or an ester or ether glucuronide of protocatechuic acid. Since the amide group of *p*-hydroxybenzamide has been shown to be resistant to hydrolysis in the rabbit, it seems probable that protocatechuic acid amide is first formed, so that the nature of the conjugates excreted will depend on the stability of this amide *in vivo*.

Isolation of 4-carbamylphenylglucuronide

p-Hydroxybenzamide was administered to each of eight rabbits at a dose level of 1 g./kg. and the urine passed during the subsequent 24 hr. (3 l.) was collected and acidified with glacial acetic acid. Excess of saturated normal lead acetate solution was added and the resulting precipitate discarded. The filtrate was made alkaline by addition of ammonia and an excess of saturated basic lead acetate solution added. The precipitate was isolated, suspended in water (1 l.) and decomposed with H_2S. The lead sulphide was filtered off and the filtrate evaporated to 100 ml. under reduced pressure at 50–60°. An excess of solid barium carbonate was stirred in, the mixture filtered and the residue washed with water. The combined filtrate and washings were precipitated by adding 5 vol. 96 % ethanol (yield of crude barium salt, 20 g.).

This was dissolved in water and decolorized with charcoal. The filtrate crystallized on evaporation. The crystals obtained were too soluble to be recrystallized from water and were purified by dissolving in aqueous ethanol and adding 96 % ethanol to the solution. On chilling, rosettes of fine white needles separated (yield, 15 g.). These did not reduce Benedict's solution without preliminary hydrolysis with 2N-HCl and melted at 299–300° with decomposition, $[\alpha]_D^{20°} = -78°$ (water). Found: C, 38·4; H, 4·0; N, 3·2; Ba, 17·1; glucuronic acid (after 1 hr. hydrolysis with 2N-HCl), 46·9; H_2O, 3·6 %. The barium salt of 4-carbamylphenylglucuronide. $C_{26}H_{28}O_{16}N_2Ba, 2H_2O$ requires C, 39·2; H, 4·0; N, 3·5; Ba, 17·2; glucuronic acid, 48·7; H_2O, 4·5 %.

p-Hydroxybenzoic acid was isolated from the compound by hydrolyzing with 2N-HCl and subsequent ether extraction. The presence of protocatechuic acid in the mixture obtained by hydrolyzing crude specimens of the barium salt suggests that it is excreted as a glucuronide in a way similar to *p*-hydroxybenzamide.

The free glucuronide was prepared from a solution of the barium salt by the careful addition of 2N-sulphuric acid until only a slight excess of barium remained. The barium sulphate formed was filtered off and the filtrate carefully evaporated. The glucuronide separated as large needles which were recrystallized from water (yield, 10 g.). It did not reduce Benedict's solution and melted at 212° with decomposition, $[\alpha]_{20°}^{D} = -93°$ (water). (Found: C, 50·2; H, 4·9; N, 4·2; glucuronic acid, 61·3 %; equivalent (by titration), 308; ash, nil. $C_{13}H_{15}O_8N$ requires C, 49·9; H, 4·8; N, 4·5; glucuronic acid, 62·0 %; equivalent, 313.)

DISCUSSION

Table 4 summarizes the quantitative results presented above. For the purpose of these calculations it is assumed that the glucuronides and ethereal sulphates formed are insoluble in ether, so that the ether-soluble acid estimated does not include these

Table 4. *Metabolites of* p-*hydroxybenzoic acid and its amide in rabbit urine after administration*

Dose level (g./kg.)	Percentage of dose excreted as: *p*-Hydroxybenzoic acid	*p*-Hydroxyhippuric acid	Total ether-soluble acid	Ester glucuronide	Ether glucuronide	Ethereal sulphate	Approx. total % accounted for
			Acid				
0·1	35	31	66	16	19	4	105
0·25	45	20	65	5	10	7	87
0·5	62	3	75	4	6	7	92
1·0	52	21	73	6	12	5	96
1·5	44	20	64	7	8	5	84
			Amide				Approx. % hydrolyzed
0·1	1	1	2	Nil	15	35	2
0·25	Nil	Nil	Nil	Nil	15	23	Nil
0·5	4	2	7	Nil	15	17	7
1·0	2	2	3	1	24	17	4
1·5	2	1	5	1	22	14	6

conjugates. Control experiments showed that the glucuronides, at any rate, are only sparingly soluble in ether. The conjugation of protocatechuic acid is also neglected, for the amount formed is very small, so that any errors due to the possibility that its two hydroxyl groups may be conjugated with sulphuric acid (Baumann & Herter, 1877; Preusse, 1878) or with glucuronic acid (Marfori, 1897) are negligible. It is clear that the presence of a hydroxyl group in the *para* position stabilizes the amide group biologically, for the rabbit, which is able to hydrolyze benzamide (Bray *et al.* 1946*a*), cannot hydrolyze *p*-hydroxybenzamide. This is shown by the difference in the ethereal sulphate conjugation of the acid and amide, by the virtual absence of extra ether-soluble acid from the urine of rabbits receiving the amide, and by the absence of extra ester type glucuronide after amide feeding. The fact that more protocatechuic acid, chiefly in the form of a conjugate, is formed from *p*-hydroxybenzamide than from the acid is another indication that the two compounds are metabolized differently. These observations are supported by the *in vitro* experiments which show that rabbit liver extracts are able to hydrolyze the amide grouping in several amides, including benzamide, but not in *p*-hydroxybenzamide. This is an interesting example of change in response occasioned by slight structural change.

The oxidation product isolated is the one which would have been expected by analogy with the oxidation of other disubstituted aromatic compounds into which a hydroxyl group is introduced. It would appear to be a general rule that when *para* or *meta* substituted amino or hydroxy compounds are oxidized in this way the new hydroxyl group is introduced *ortho* to the amino or hydroxyl group. In the *meta* compounds the new group is *para* to the other (i.e. not amino or hydroxyl) substituent group and not *meta* as in the *para* substituted compounds, so that in either case the product is a 3:4-disubstituted compound. Examples of such oxidations are given in Table 5. In the two *ortho* substituted hydroxy compounds quoted, the new hydroxyl group is inserted *para*, not *ortho*, to the existing hydroxyl group. It seems that *ortho* amino compounds behave still differently, since Jaffe & Hilbert (1888) found that *o*-acetoluidide in dogs gave rise to a benzoxazolone derivative, presumably derived from 2-amino-3-hydroxytoluene. It will be of interest to see whether Williams (1945) can substantiate his suggestion that *o*-aminobenzenesulphonamide is oxidized to the vicinally substituted 2-amino-3-hydroxybenzenesulphonamide in the rabbit. We are investigating *o*- and *m*-hydroxybenzoic acids and their amides and the aminobenzoic acids and amides. These studies may provide further examples of this type of oxidation.

SUMMARY

1. A study of the metabolism of *p*-hydroxybenzoic acid and its amide in the rabbit has been made.

2. Estimation of the excretion of ether-soluble acid (*p*-hydroxybenzoic and *p*-hydroxyhippuric acid fractions), ester and ether type glucuronides and ethereal sulphate has provided evidence that the amide group in *p*-hydroxybenzamide is not hydrolyzed to any appreciable extent *in vivo*.

3. This conclusion is supported by enzyme studies.

4. Metabolites of the two compounds, which have been isolated from the urine of rabbits receiving them, are *p*-hydroxyhippuric acid from the acid and protocatechuic acid and 4-carbamylphenylglucuronide from the amide.

We are indebted to the Royal Society for a Government Grant which defrayed part of the cost of this work. The enzyme studies were carried out with the co-operation of Dr Sybil P. James. The micro-analyses were carried out by Drs Weiler and Strauss, Oxford.

Table 5. *Configuration of hydroxy-compounds isolated as metabolites of hydroxy- and amino-compounds*

Compound	Oxidation product	Animal or enzyme	Reference
p-Hydroxybenzoic acid	3:4-Dihydroxybenzoic acid	Rabbit	Present communication
p-Hydroxybenzamide	3:4-Dihydroxybenzoic acid	Rabbit	Present communication
p-Cresol	3:4-Dihydroxytoluene*	Tyrosinase	Pugh & Raper (1927)
p-Hydroxyphenylalanine (tyrosine)	3:4-Dihydroxyphenylalanine	Tyrosinase	Raper (1926)
p-Hydroxybenzenesulphonamide	3:4-Dihydroxybenzenesulphonamide	Rabbit	Williams (1941)
p-Aminobenzenesulphonamide	4-Amino-3-hydroxybenzenesulphonamide	Rabbit	Thorpe, Williams & Shelswell (1941); Williams (1946)
m-Cresol	3:4-Dihydroxytoluene*	Tyrosinase	Pugh & Raper (1927)
m-Aminobenzenesulphonamide	3-Amino-4-hydroxybenzenesulphonamide	Rabbit	Dobson & Williams (1946)
o-Hydroxybenzoic acid	2:5-Dihydroxybenzoic acid	Rat	Lutwak-Mann (1943)
o-Cresol	2:5-Dihydroxytoluene	Dog	Preusse (1881)
o-Acetoluidide	Derivative of 2-amino-3-hydroxytoluene	Dog	Jaffe & Hilbert (1888)
o-Aminobenzenesulphonamide	2-Amino-3-hydroxybenzenesulphonamide (?)	Rabbit	Williams (1945)

* Preusse (1881) did not detect these oxidation products in the dog.

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The Action of Formaldehyde on the Cystine Disulphide Linkages of Wool

2. THE CONVERSION OF SUBFRACTION A OF THE COMBINED CYSTINE INTO COMBINED LANTHIONINE AND DJENKOLIC ACID AND SUBFRACTION B INTO COMBINED THIAZOLIDINE-4-CARBOXYLIC ACID

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Fraction (A+B) of the combined cystine of wool reacts with $NaHSO_3$ to give cysteine and *S*-cystinesulphonate side-chains: those derived from subfraction A revert to disulphide when the bisulphited wool is rinsed with water, whereas the cysteine and *S*-cysteinesulphonate side-chains derived from subfraction B are water-stable (Elsworth & Phillips, 1941; Middlebrook & Phillips, 1942*a*). When wool is treated at 70° with solutions of formaldehyde at a pH between 5 and 7, half of the sulphur of subfraction B is converted into combined thiazolidine-4-carboxylic acid (Middlebrook & Phillips, 1942*b*). Since subfraction B reverts to disulphide when this formalized wool is boiled with H_3PO_4, it is possible that the formaldehyde condenses with the thiol group produced by the hydrolysis of the disulphide group (—SS— $\rightleftharpoons$ —SH + —SOH), but leaves the sulphenic acid group unchanged. The sulphenic acid group may condense with an adjacent imino group forming an *iso*thiazole ring (McClelland & Warren, 1930; Middlebrook & Phillips, 1942*b*), but may be liberated when the wool is boiled with H_3PO_4.

Stoves (1943) boiled keratin fibres with solutions of formaldehyde and from an examination of their load-extension curves suggested that some of the cystine cross-linkages had been converted into djenkolic cross-linkages (:$CHCH_2SCH_2SCH_2CH$:). We have treated wool under similar conditions to those used by Stoves (1943), but find that it does not contain combined djenkolic acid; subfraction A is converted into lanthionine and subfraction B into thiazolidine-4-carboxylic acid. On the other hand, when fraction (A+B) is reduced with thiolacetic acid (Middlebrook & Phillips, 1942*b*), and the reduced wool is treated with formaldehyde, combined djenkolic acid arises from subfraction A, whilst subfraction B is converted into combined thiazolidine-4-carboxylic acid.

When wool in which fraction (A+B) has been reduced is treated with methylene di-iodide, only subfraction A is converted into combined djenkolic acid; both subfractions are, however, cross-linked, when the reduced wool is treated with ethylene dibromide.

METHODS

Materials and analytical methods. The wool used (80's quality; total-S, 3·65; disulphide-S, 3·06%) was in the form of a knitted fabric which had been cleaned with soap, rinsed in water, extracted with ethanol and finally rinsed in water.

Unless otherwise stated, the treated and untreated wools were hydrolyzed under reflux in 5N-HCl for 4 hr. The

disulphide and thiol-S contents were determined by Shinohara's methods (1935*a*, *b*, *c*), using a Spekker absorptiometer. The cystine and djenkolic, lanthionine and thiazolidine-4-carboxylic acids were determined qualitatively by the paper chromatographic technique of Consden, Gordon & Martin (1944, 1946). The presence or absence of tyrosine and other amino-acids was noted. For use as standards, djenkolic acid (du Vigneaud & Patterson, 1936) and thiazolidine-4-carboxylic acid (Ratner & Clarke, 1937) were synthesized, whilst lanthionine was isolated from alkali-treated wool. Thiazolidine-4-carboxylic acid was determined from the colour increase due to the slow decomposition of the acid to cysteine by the $NaHSO_3$ used in the disulphide-S determinations (Middlebrook & Phillips, 1942*b*). Total S was determined by the Benedict-Denis method (Barritt, 1934) and formaldehyde by distilling the wools with 0·1 M-H_3PO_4, the formaldehyde being trapped in a solution of $NaHSO_3$ (Nitschmann & Hadorn, 1941). All the wools, described as conditioned, had been exposed to a controlled atmosphere of 21° and 70% R.H. until they attained constant weight. The analyses are all expressed as a percentage of the weight of the anhydrous wool.

RESULTS

Reaction between wool and formaldehyde at different pH *values*

In each experiment the wool was treated, under reflux, with 50 times its weight of 1% (w/v) formaldehyde. Acid, neutral and alkaline solutions of formaldehyde were used as follows: (i) at 100° and pH 1·00 for 1 hr., in dilute HCl containing Na acetate in 0·2 M concentration; (ii) at 100° and pH 6·65 for 1 hr. with 0·02 M-Na_2HPO_4 as buffer; (iii) at 70° and pH 6·7 for 48 hr., the same buffer being used; (iv) at 100° and pH 10·12 (falling during the treatment to pH 9·40) for 1 hr. with 0·05 M-borax and NaOH as buffer.

(*a*) *Acid and neutral treatments* (*i*), (*ii*) *and* (*iii*). These caused closely similar changes in the wool, the disulphide-S falling in each experiment to 2·10%. The 'colour increases', due to decomposition of the thiazolidine-4-carboxylic acid by the $NaHSO_3$ added in the disulphide-S determination, were equivalent to from 0·60 to 0·54% cysteine-S, corresponding to approximately half the observed loss of disulphide-S (0·96%). Thiazolidine-4-carboxylic acid was found in all three hydrolysates by one-dimensional paper chromatograms using collidine: it gave a yellow 'spot' with ninhydrin which changed to purple, like the spot given by proline. The acid had a high R_F value which exceeded that of tyrosine. By a two-dimensional paper chromatogram, the three hydrolysates were found to be free from lanthionine and tyrosine.

Samples (0·5 g.) of the pH 1·0-treated and pH 6·65-treated wools were distilled with 0·1 M-H_3PO_4 until the volume of the residual acid in the flask was 10 ml. The formaldehyde evolved was equal to 0·70% of the pH 1·0-treated wool and to 1·45% of the pH 6·65-treated. The two acid-treated wools were rinsed in water, conditioned and analyzed. In each case, their disulphide-S contents were found to be very close to 3·06%, indicating that the formaldehyde reaction had been reversed completely. This was confirmed by a one-dimensional chromatogram of the hydrolysate of the formaldehyde-free pH 1·0-treated wool which showed the absence of thiazolidine-4-carboxylic acid: a two-dimensional chromatogram showed the absence of lanthionine.

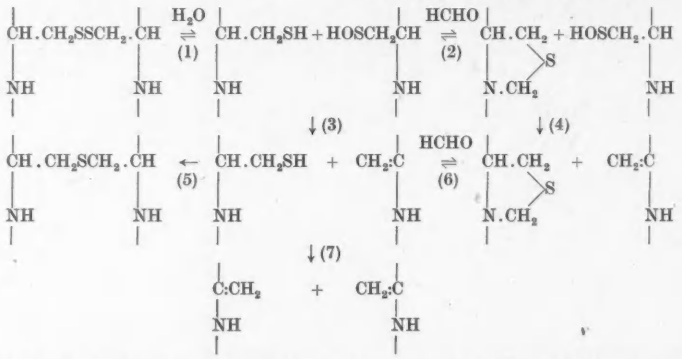
(*b*) *Alkali treatment.* During treatment, the total S of the wool decreased by 1·0% and the disulphide-S to 0·2%. The hydrolysate of the wool gave a 'colour increase' corresponding to 0·54% cysteine-S. Since, when one S atom of a disulphide group reacts with formaldehyde, the other is converted into a sulphenic acid group, the total disulphide-S reacting with formaldehyde was 1·08%, leaving 1·94% disulphide-S which lost 1·0% S to give 1% lanthionine-S. A one-dimensional chromatogram confirmed the presence of thiazolidine-4-carboxylic acid, whilst the presence of lanthionine and the absence of cystine and tyrosine was confirmed by a two-dimensional chromatogram.

Although the significance of the numerical values of these determinations is decreased because of small, but unknown, losses of wool substance during the treatments, the results show that the reaction between cystine-S and alkaline formaldehyde differs markedly from that between cystine-S and either acid or neutral formaldehyde. In boiling acid or neutral solutions of formaldehyde, subfraction B undergoes reactions (1) and (2) (see below) yielding combined thiazolidine-4-carboxylic acid. In the absence of formaldehyde, boiling wool in a solution of pH 10·12 converts fraction (A + B) into lanthionine by reactions (1), (3) and (5), and by reactions (1), (3) and (7) partially converts fraction (C + D) into combined α-aminoacrylic acid (Lindley & Phillips, 1945). The present results suggest that in boiling alkaline solution at pH 10·12, even in the presence of formaldehyde, fraction (A + B) mainly undergoes the normal alkaline decomposition and yields lanthionine. Some combined thiazolidine-4-carboxylic acid is also produced and may arise from subfraction B by reactions (1) and (2) or from fraction (C + D) by reactions (1), (3) and (6).

If thiazolidine-4-carboxylic acid is produced from subfraction B in this manner, then alkaline decomposition of the sulphenic acid radical may occur by reaction (4) yielding combined α-aminoacrylic acid: the end-products of the reaction may therefore be the same as those arising from fraction (C + D) by reactions (1), (3) and (6).

Since we have found that the cystine-S of wool formalized under neutral and acid conditions can be regenerated by distilling the wool with 0·1 M-H_3PO_4, we conclude such treatment reverses reactions (2)

and (1). This provides a method of detecting whether reactions (4) and (6) take place when wool is treated with alkaline formaldehyde, since hydrolysis of the product of these reactions cannot yield cystine, but may yield combined cysteine, or alternatively, combined lanthionine, if the hydrolysis is followed by reaction (5).



The wool which had been formalized in alkaline solution was therefore distilled with 0·1 M-H_3PO_4. It yielded 2·32 % formaldehyde and when hydrolyzed subsequently was found to contain 0·54 % cystine-S, but no thiol-S. A one-dimensional chromatogram of the hydrolysate showed the absence of thiazolidine-4-carboxylic acid and a two-dimensional chromatogram the presence of lanthionine: the cystine spot was very faint.

During the distillation with 0·1 M-H_3PO_4, 0·34 % cystine-S (0·54–0·20) was reformed so that 0·37 % of cysteine-S (0·54–0·17) is unaccounted for. This may have been lost from subfraction B, when the formalized wool was hydrolyzed, owing to the occurrence of reaction (4) during the alkaline treatment and subsequent formation of lanthionine by reversal of reaction (6) followed by reaction (5). Alternatively, the missing thiol originated from fraction (C + D) and was converted into lanthionine during the hydrolysis, again by reversal of reaction (6) followed by reaction (5).

Reaction between reduced wool and formaldehyde before and after supercontraction

Before supercontraction. To determine whether djenkolic acid cross-linkage could be produced in wool in which fraction (A + B) had been reduced to thiol groups (Middlebrook & Phillips, 1942*b*), a wool was reduced by treating it at 37° for 17 hr. with 50 times its weight of M-thiolacetic acid titrated with NaOH to pH 5·2 (Patterson, Geiger, Mizell & Harris, 1941). The reduced wool was rinsed thoroughly with distilled water, then with ethanol and finally with water. After conditioning, it contained 2·28 % thiol-S and 0·78 % disulphide-S.

As in the previous experiment (i) with ordinary wool, it was treated with formaldehyde at pH 1·0. On analysis it was then found to be free from thiol-S, to contain 0·76 % disulphide-S and to give a 'colour increase' equivalent to 1·32 % thiol-S. In the previous experiments, a 'colour increase' equivalent to 0·60 % thiol-S corresponded with the reaction of formaldehyde with 0·96 % disulphide-S or the formation of thiazolidine-4-carboxylic acid from 0·48 % thiol-S. The observed 'colour increase' of 1·32 % is therefore equivalent to 1·06 % S combined as thiazolidine-4-carboxylic acid, making with the 0·76 % disulphide-S remaining unchanged, 1·82 % S accounted for out of the original 3·06 %, so that 1·24 % S has become linked in some other manner.

The treated wool was distilled with 0·1 M-H_3PO_4 to remove the combined formaldehyde and then hydrolyzed when it was found to contain 0·59 % thiol-S and 0·71 % disulphide-S. In addition the hydrolysate gave a 'colour increase' equivalent to 0·49 % thiol-S, indicating that the removal of the formaldehyde had been incomplete. A two-dimensional chromatogram of the acid hydrolysate showed that it contained djenkolic acid, so that this acid may therefore account for the missing 1·24 % S.

Djenkolic acid was distilled with 0·1 M-H_3PO_4 until the volume of the residual acid was 10 ml. This residual acid was free from thiol and disulphide-S and gave no 'colour increase' on standing with $NaHSO_3$ at pH 5. Although stable to 0·1 M-H_3PO_4, djenkolic acid was decomposed when heated with concentrated H_2SO_4 into formaldehyde, cysteine and cystine.

After supercontraction. When wool fibres are treated with reagents that break the disulphide cross-linkages of fraction (A + B), they almost invariably assume, when boiled with water, a length about 30 % less than their normal length. This contraction was called supercontraction by Astbury & Woods (1933), and has been assumed to be accompanied by molecular reorientation (Speakman, 1936) which may alter the relative spacial positions of the

two S atoms and hence restrict the types of reaction in which they can participate. Since we have found that thiolacetic acid-reduced wool supercontracted when boiled for 5 min. in dilute acetic acid (pH 2·8), we have compared the reactions of the reduced, and the reduced and supercontracted wool with formaldehyde. The two wools were each treated at 50° for 48 hr. in 50 times their weight of 1 % formaldehyde, buffered to the pH values indicated in Table 1. Before supercontraction the reduced wool contained thiol-S, 2·28 and disulphide-S, 0·78 %: after supercontraction its thiol-S content was 2·06 and its disulphide-S content 0·75 %. Apart from the two major differences, indicated as footnotes to Table 1, the two wools, after treatment with formaldehyde, gave the same analyses.

Table 1. *Analyses of reduced and supercontracted wools after treatment with* 1 % *formaldehyde at different* pH *values*

pH of formaldehyde solution	Thiol-S (%)	Disulphide-S (%)	Thiol-S calc. from 'colour increase' (%)
1·0	0·1	0·77	1·33
6·6	0·1	0·89	1·48*
8·0	0·05	0·96†	1·47

* Reduced and supercontracted wool gave 1·37 %.
† Reduced and supercontracted wool gave 0·76 %.

The identity in chemical analysis of the two treated wools persisted after they had both been distilled with 0·1 M-H_3PO_4; the reduced wool contained 0·62 % thiol-S, 0·97 % disulphide-S and its acid hydrolysate gave a 'colour increase' of 0·38 %, the corresponding analyses for the reduced and supercontracted wool being 0·51, 0·92 and 0·35 %.

The reaction between reduced wool and methylene di-iodide and ethylene dibromide

A fully reduced wool (Patterson *et al.* 1941) was treated for 4 hr. at 50° with 0·0005 mol. methylene di-iodide in 0·1 M-phosphate buffer (20 ml.) at pH 8·0–7·2 and then rinsed thoroughly and conditioned. The treated wool contained 1·24 % thiol-S and 0·98 % disulphide-S, and the hydrolysate gave no colour increase in the presence of $NaHSO_3$. The analysis of the wool was unchanged (thiol-S, 1·30 %; disulphide-S, 1·02 %) by distillation with 0·1 M-H_3PO_4. A one-dimensional chromatogram of the hydrolysate of this H_3PO_4-treated wool confirmed the presence of cystine and djenkolic acid and the absence of thiazolidine-4-carboxylic acid.

In order to demonstrate that the thiol-S in the reduced wool which did not react with methylene di-iodide would react with formaldehyde, reduced wool which had been treated with methylene di-iodide was heated under reflux with 1 % formaldehyde at pH 1·0 for 1 hr. When analyzed, it was found to be free from thiol-S, its disulphide-S was unchanged (0·99 %), but its hydrolysate in the presence of $NaHSO_3$ showed a 'colour increase' corresponding to 1·30 % thiol-S. The thiol groups in the reduced wool which failed to react with methylene di-iodide had therefore reacted with formaldehyde to give thiazolidine-4-carboxylic acid; hence, it is probable that they were produced mainly from subfraction B.

A comparison was then made between the reaction of reduced wool with methylene di-iodide and ethylene dibromide. A purified virgin Cape wool (Middlebrook & Phillips, 1942*a*) was reduced in 100 times its weight of M-thiolactic acid for 17 hr. at pH 5·0 and 32°. After rinsing in several changes of water, it contained 2·07 % thiol-S. Samples (*c.* 0·5 g.) of this reduced wool were treated, as set out in Table 2, in 50 ml. of buffer (M-Na_2HPO_4 with H_3PO_4 for pH 8·0 and M-Na acetate with acetic acid for pH 5·4) to which different quantities of the dihalides had been added. The thiol-S contents of the treated wools were then redetermined.

Table 2. *The reaction between reduced wool* (*c.* 0·5 *g.*) *and methylene di-iodide and ethylene dibromide*

Dihalide (g.)	Buffer (pH)	Treatment: Temp. (°)	Treatment: Time (hr.)	Residual SH, as S (%)
Methylene di-iodide				
0·3	8·0	32	17	0·69
0·3	8·0	32	48	0·74
0·6	8·0	50	18	0·65
0·3	5·4	32–55	66	0·53
Ethylene dibromide				
0·2	5·4	32–55	66	0·17
0·2	8·0	32	17	0·17

From Table 2 it will be seen that whereas a portion of the thiol groups in the reduced wool failed to react with methylene di-iodide, practically all the thiol groups reacted with ethylene dibromide.

The greater ease with which the thiol groups derived from subfraction A, as compared with those of subfraction B, can be cross-linked with methylene di-iodide, provides a method of comparing the relative rates at which subfractions A and B are reduced by thiolacetic acid. Samples of virgin Cape wool (Middlebrook & Phillips, 1942*a*) were reduced with 100 times their weight of M-, 0·2 M- and 0·05 M-thiolacetic acid at pH 5·0 for 18 hr. at 32°. After they had been rinsed to remove thiol and dithiolacetic acid, part of each sample was hydrolyzed to determine the thiol-S content and part (*c.* 0·5 g.) treated in 50 g. of M-phosphate buffer at pH 8·0 and 32° containing 0·3 g. methylene di-iodide for 22 hr. Their thiol-S contents were then redetermined and the

loss of thiol-S calculated and returned as djenkolic acid-S (Table 3). On the assumption that the thiol-S which did not react belongs to subfraction B and the thiol-S which was converted into djenkolic acid-S to subfraction A, the results suggest that the two subfractions are reduced at rates roughly dependent on the relative proportions of the two subfractions in the wool.

Table 3. *Reaction between fully and partially reduced wools and methylene di-iodide*

Reduced wools	Reduced wools after CH_2I_2	
S as SH (%)	S as SH (%)	S as djenkolic-S (%)
2·07	0·68	1·39
1·39	0·46	0·93
0·58	0·32	0·26

DISCUSSION

The simplest explanation of the observed differences in the reactivities of subfractions A and B would appear to be that when the S atoms of fraction (A + B) are separated by chemical reaction, those of the disulphide groups of subfraction A do not move so far apart as do those of subfraction B. This would explain why subfraction A can be transferred into lanthionine more readily than subfraction B (Lindley & Phillips, 1945) and why it yields djenkolic acid with methylene di-iodide and formaldehyde, whereas subfraction B reacts with ethylene dibromide but not with methylene di-iodide, and yields thiazolidine-4-carboxylic acid with formaldehyde.

Other observations on oxidation reactions depending on the availability of two thiol groups in close proximity suggest that some of the thiol groups of reduced wool, which have arisen from fraction (A + B), can move outside reactive distance (Blackburn, Middlebrook & Phillips, 1942). For example, the thiol groups which persist after treatment of reduced wool with methylene di-iodide in a buffer at pH 5 do not give the nitroprusside reaction. Further, about 0·7% of cysteine-S remains unoxidized when reduced wool, immersed in a buffer at pH 8, is treated with oxygen. On the other hand, all such thiol groups react readily with methyl iodide (Harris, Mizell & Fourt, 1942). In addition, a bisulphited and rinsed wool, which contains thiol and *S*-cysteinesulphonate groups derived from subfraction B, can be readily methylated (Blackburn, Consden & Phillips, 1944).

The variations in the ease with which free thiol groups in wool can be cross-linked or oxidized is allied to the phenomenon of the 'unmasking' of thiol groups during the denaturation of cystine-containing proteins (Neurath, Greenstein, Putman & Erickson, 1944). Neurath (1938) has suggested that this 'unmasking' takes place because the protein assumes a more flexible configuration which permits closer approach of the thiol groups of adjacent chains and thus facilitates their oxidation to disulphide groups.

The evidence that thiol groups exist in the native proteins is not so definite as with reduced wool. Anson (1940) showed that those of native egg albumin, although unreactive towards nitroprusside, would react with iodoacetamide, but Rosner (1940) could not cause them to react with iodoacetate until the albumin had been denatured. Iodoacetamide and iodoacetate do not, however, react with equal ease with the thiol groups of proteins, since Dr H. Lindley, in these laboratories, has found that whereas at pH 6 all the thiol groups of fraction (A + B) will react with iodoacetamide, only a portion will react with iodoacetate.

SUMMARY

1. Subfraction B of the combined cystine of wool, defined as the subfraction which gives water-stable thiol and *S*-cysteinesulphonate groups with $NaHSO_3$, reacts with formaldehyde at pH values of 1·0, 6·7 and 10·0 at 70–100° to give combined thiazolidine-4-carboxylic acid.

2. Subfraction A of the combined cystine of wool, defined as the subfraction which gives water-labile thiol and *S*-cysteinesulphonate groups with $NaHSO_3$, does not react with formaldehyde at pH values of 1·0, 6·7 and 10·0 at 70–100°, but at pH 10 is converted into combined lanthionine.

3. Wool in which fraction (A + B) has been reduced to thiol groups reacts with formaldehyde, subfraction A being converted into combined djenkolic acid and subfraction B into combined thiazolidine-4-carboxylic acid. When this reduced wool is treated with methylene di-iodide only subfraction A is cross-linked and converted into combined djenkolic acid.

4. Wool in which fraction (A + B) has been reduced to thiol groups and has then been caused to supercontract reacts with formaldehyde in the same manner as normal, reduced wool.

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The Action of Alloxan and Related Compounds on Alkaline Phosphatase

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The action of alloxan in producing degranulation and degeneration in the β-cells of the pancreatic islets is unique in many ways. The toxic action is apparently irreversible after a few minutes in which time the earliest signs of degeneration appear (Bailey, Bailey & Hagan, 1944; Goldner, 1945; Weinglass, Frame & Williams, 1945). The lesion is very specific in that the α-cells are almost wholly spared and only with larger doses do renal and hepatic necrosis appear. Goldner & Gomori (1944) showed that insulin injection did not protect the pancreas against alloxan, unlike diabetes due to anterior pituitary extracts (Young, 1937).

As general cytotoxic factors can clearly be excluded by the very specificity of the lesion, it would seem reasonable to suppose that alloxan might interfere with cell enzymes, possibly those involved in insulin synthesis. An approach to the problem could thus be made by studying the action of alloxan and the related compounds that are diabetogenic (Brückmann & Wertheimer, 1945, and others) on enzyme systems both isolated and in tissue slices.

Alkaline phosphatase has been studied here because we found early that it was strongly inhibited by alloxan itself, and it has the advantage that it can be studied quantitatively in tissue sections by a modification of the Gomori (1939) histochemical technique. Further, Gloetens (1942, 1944) showed that Zn was the prosthetic group of alkaline tissue phosphatase and Zn appears to be an integral part of the insulin molecule.

METHODS

Hog kidney phosphatase, purified by kaolin adsorption and elution into aqueous pyridine, was used for the *in vitro* experiments with a phenylphosphate substrate buffered at pH 9·4. The liberated phenol was estimated by the method of Theis & Benedict (1924). In tissue sections Gomori's method (Gomori, 1939) was used but incubation was carried out usually for 15–30 min. only and all the slides in a series were treated simultaneously so that the comparative depth of the black cobalt sulphide precipitated was a roughly quantitative index of the phosphatase activity. The $CaCl_2$ used by Gomori tended to precipitate some of the inhibitors and was advantageously replaced by the magnesium salt.

RESULTS

Table 1 summarizes the results obtained with four diabetogenic ureides and ten related compounds. Alloxan, dialuric acid, and 1-methylalloxan were all

Table 1. *Action of alloxan and related compounds on alkaline phosphatase*

	Phosphatase inhibition	Diabetogenic activity
1. Alloxan	+ + +	+
2. Dialuric acid	+ + +	+
3. 1-Methylalloxan	+ + +	+
4. Alloxantin	+ +	+
5. *iso*Dialuric acid	+	−
6. Alloxanic acid	+	−
7. Mesoxalic acid	−	−
8. Violuric acid	−	−
9. Uracil	−	−
10. Thiouracil	−	−
11. Barbiturates	−	−
12. Allantoin	−	−
13. Urea	−	−
14. Thiourea	−	−

diabetogenic and strong inhibitors of phosphatase in concentrations down to 0·001 M (Fig. 1). Alloxantin was also diabetogenic but only about two-thirds as powerful an inhibitor of phosphatase. Alloxanic acid and *iso*dialuric acid were not diabetogenic and they had a weaker inhibitory action on phosphatase; at a concentration of 0·001 M they inhibited less than 10 %. The inhibition with these two compounds was almost linear with concentration (Fig. 1), unlike the previous four compounds which gave almost cubic expressions.

Fig. 2 shows the chemical relationships of the compounds tested. All four diabetogenic and strongly phosphatase inhibiting compounds are

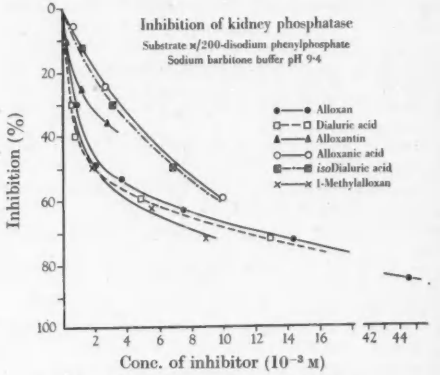

Fig. 1. Inhibition of kidney phosphatase by alloxan and related ureides.

closed ring ureides with a hydroxyl or a reactive keto group in the 5-position. Compounds such as uracil and barbituric acid without this group are inactive; if this group is blocked as by oxime formation in violuric acid all activity is lost. Alloxantin

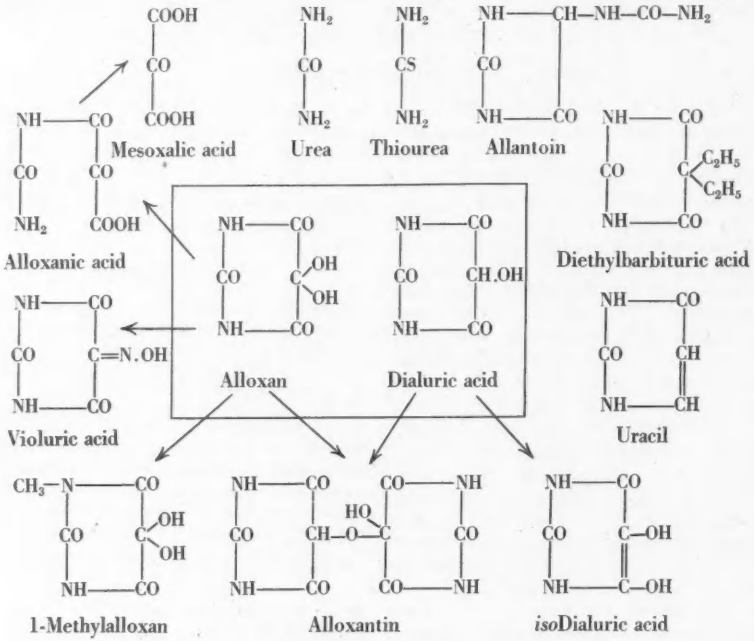

Fig. 2. The chemical relationships of the compounds tested.

illustrates this in that, as a result of the taking up of one 5-hydroxyl group in an ether linkage, only one group is free for the two rings and the phosphatase inhibitory action per ring is somewhat less than half that of alloxan. The diabetogenic activity, however, is unchanged. On opening the ureide ring as in alloxanic acid the anti-phosphatase activity declines and the power to produce diabetes is lost; complete hydrolysis to mesoxalic acid and urea removes the rest of the activity against phosphatase. *iso*Dialuric acid which has a carboxyl group replaced by a hydroxyl group is isomeric with dialuric acid and behaves like alloxanic acid.

This inhibition of phosphatase has been demonstrated in kidney sections and these have shown an inhibition comparable to that in the test tube. Plate 3 shows that with increasing concentrations of alloxan there is a progressive inhibition of both nuclear and cytoplasmic phosphatase in the kidney tubule. This inhibition was reversible as shown by washing the section and then incubating in substrate alone, when a normal amount of phosphatase appeared. The same phenomenon has been shown *in vitro* by incubating the concentrated enzyme with 0·06 M-alloxan buffered at pH 9·4 at 37° for 1 hr., then diluting with buffer and incubating with substrate, giving a final concentration of 0·0003 M-alloxan. The enzyme did not differ in activity from an unincubated enzyme-alloxan mixture prepared in the same way and was only 5% less active than enzyme incubated with buffer alone. It is clear that alloxan does not destroy phosphatase.

Goldner (1945), Goldner & Gomori (1945) and Archibald (1945) suggest that alloxan but not dialuric acid or alloxantin are very rapidly destroyed at neutral or alkaline pH. But alloxan and dialuric acid behave similarly in their action, which is more marked than that of alloxanic acid, their supposed end-product. We have found the inhibitory action of alloxan unaffected by the presence of plasma or whole blood.

The inhibition of kidney phosphatase was next tested *in vivo* by injecting rats and rabbits with alloxan and assaying their kidney phosphatase both quantitatively and histochemically a day or more later. In every case islet degeneration was present in the pancreas. The assays revealed:

Phosphatase content of kidneys of control rats
$= 41 \pm 3$ King-Armstrong units/g.

Phosphatase content of kidneys of rats given 100 mg. alloxan/kg.
$= 39 \pm 5$ King-Armstrong units/g.

These differences are not significant.

No difference was to be seen in the kidneys of the control and treated rats. In the pancreas the degenerated islets had less phosphatase than the normal, which was not surprising in view of the degree of shrinkage and degeneration the islets had undergone.

We have shown further that the inhibition by alloxan was independent of the concentration of Mg^{++} ions present.

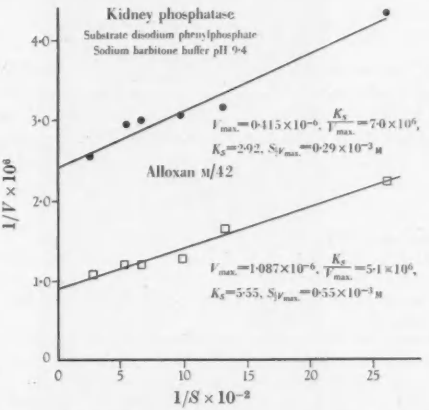

Fig. 3. Enzyme-substrate dissociation constants plotted according to the method of Lineweaver & Burk.

The dissociation constant of enzyme-substrate complex has been studied by Lineweaver & Burk's (1934) modification (Fig. 3) of the Michaelis-Menten (1913) method. In the presence of 0·024 M-alloxan the maximum velocity of the reaction was reduced from 1·09 μmol. hydrolyzed/min. to 0·42 μmol./min., whereas the slope which represents the ratio of dissociation constant K_s to the maximum velocity $V_{max.}$ only changed from $5{\cdot}1 \times 10^6$ to $7{\cdot}0 \times 10^6$. These findings indicate that the inhibitory action of alloxan is mainly non-competitive.

DISCUSSION

Alloxan and three other diabetogenic ureides have been shown to be strong inhibitors of alkaline phosphatase in the concentrations required to produce diabetes. This is quite independent of the power of alloxan in concentrated solutions to deaminate and decarboxylate amino-acids, as in the low concentrations used in these experiments alloxan has no such action. Brückmann & Wertheimer (1945) tested ninhydrin and sodium β-napthoquinone sulphonate both of which also oxidatively deaminate and decarboxylate α-amino-acids and found them not to be diabetogenic. Man, Kartin, Durlacher & Peters (1945) found that alloxan combines with the sulphydryl group of enzymes, but again only in high concentrations. The action of alloxan and related compounds on phosphatase described in this paper is a reversible non-competitive inhibition, the nature

of which might conceivably be due to interaction with the zinc prosthetic group of the enzyme. Perhaps some interference with zinc transfer in insulin synthesis may account for the action of alloxan on the β-cells of the pancreatic islets.

SUMMARY

1. Four diabetogenic ureides were examined for their power to inhibit alkaline phosphatase and were all highly active. Of ten related compounds examined, two were poor inhibitors of phosphatase although not diabetogenic and non-inhibiting for phosphatase. The anti-phosphatase activity seems to reside in a free 5-hydroxyl group.

2. This action on phosphatase has been demonstrated in the presence of plasma and blood and in tissue sections. It is reversible, non-competitive and unaffected by magnesium.

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The Estimation of Histidine In Urine and its Excretion by Normal and Pregnant Women

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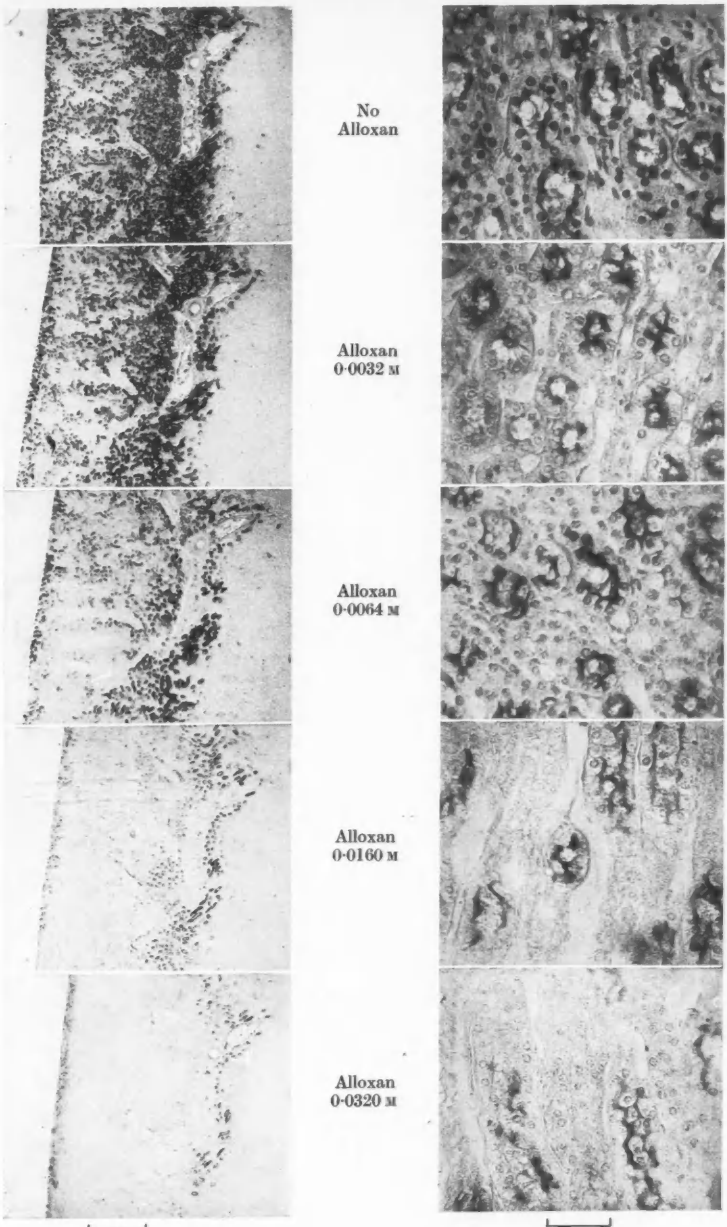
The urinary excretion of histidine has been a matter of interest since Voge (1929) demonstrated an increase in the excretion during normal pregnancy. This finding has been confirmed (Kapeller-Adler, 1934; Niendorf, 1940; Racker, 1941; Langley, 1941; O'Brien & Quelch, 1943; Dawson, 1944), together with the fact that the excretion drops in pre-eclamptic toxaemia. These results have been obtained by examination of a few urine samples from a considerable number of individuals, but in studying the metabolism of histidine it is of interest to examine the excretion for the duration of the pregnancy in order to ascertain whether any definite trends in histidine excretion are observed. The results obtained in the course of two pregnancies are presented in this paper together with some observations on the excretion of histidine by normal women at different times during the menstrual cycle. The latter work was undertaken as a result of a suggestion by Boxer & Kapeller-Adler (1937) that the gonadotrophic hormones inhibited the activity of liver histidase thus giving rise to the increased excretion of histidine during pregnancy. This conception would be strengthened if it could be demonstrated that normal women showed a rise in histidine excretion at the time of greatest gonadotrophic hormonal activity in the menstrual cycle, although Boxer & Kapeller-Adler found the rise in excretion occurred approximately at the time of the first occurrence of a positive Ascheim-Zondek test which suggested that a minimal level of circulating gonadotrophic hormone was essential to affect the histidine excretion.

The method of estimation of urinary histidine is based on a colour reaction described by Knoop (1908) and consists of a bromination of the histidine, removal of excess bromine and heating the product at 100° when a characteristic colour develops. The method as applied by Armstrong & Walker (1932) and Kapeller-Adler (1934) has been criticized on the grounds of interference by the urea, urates, phosphates and pigments of the urine (Racker, 1940;



Inhibition of phosphatase in kidney sections by alloxan.

Langley, 1941). It has been found that two distinct pigments can be produced according to the pH at which the heating is carried out and a technique has been devised in which the pH is controlled and errors due to other causes have been overcome.

METHODS

The estimation of histidine in urine

Reagents. (*a*) 10% (v/v) H_2SO_4, (*b*) N- and 0·1 N-$KMnO_4$, (*c*) bromine reagent: bromine (1 ml.) is added to 100 ml. glacial acetic acid and the volume made up with distilled water to 200 ml. This reagent is kept in the dark and renewed every 3 days. (*d*) 0·5% phenol and (*e*) a fresh standard solution of histidine hydrochloride containing 1 mg./ml.

Procedure. To a series of four reaction tubes graduated at 10 ml., varying amounts of the standard histidine hydrochloride solution are added and the same volume of the urine added to each tube, this volume being such that the final amounts of histidine present lie within the range 0·8–1·6 mg. (the volume required is determined by a previous rough estimation). Sulphuric acid, 0·5 ml. of 10% (v/v), is added and the contents diluted to approximately 5 ml. with distilled water. Potassium permanganate is then added drop by drop to each tube until a pink colour, permanent for 30 sec., is obtained. With concentrated urines, N-$KMnO_4$ must be added initially until the pink colour is permanent for approximately 20 sec. and then the treatment completed with 0·1 N-$KMnO_4$; with weaker urines only the latter is necessary. Bromine reagent (0·5 ml.) is then added to each tube and the contents allowed to stand for 5 min. For the majority of urines this amount has been found sufficient, but the tube containing the greatest concentration of histidine may be examined at 2 min. intervals for the presence of excess bromine by means of starch-iodide paper, and if none is present a further 0·5 ml. bromine reagent should be added to all tubes. Excess bromine is removed at the end of the 5 min. period by addition of 0·5 ml. of 0·5% phenol. The volumes are then adjusted to 10 ml. and the tubes heated in the dark in a boiling water bath for 5 min., cooled and the volumes again adjusted to 10 ml. The histidine value represented by the colour developed is determined either from a standard curve determined at the same time under identical conditions with the Hilger 'Spekker' photoelectric absorptiometer using Ilford spectrum blue filter or by direct comparison with the colour developed from known amounts of histidine with a Klett Bio-colorimeter. When the Spekker photoelectric absorptiometer is used, a urine blank is included which receives identical treatment except that 50% acetic acid is substituted for the bromine reagent, and the relationship between the optical density of the reaction mixture and histidine concentration was found to be represented by a straight line between the limits of 0·8–1·6 mg. histidine/10 ml.

A typical urine estimation is reproduced in Table 1. Where the recovery of added histidine differs markedly from the theoretical the urine value is obtained by calculation but in the majority of instances the recovery is almost theoretical and the value can be obtained from the graph.

Table 1. *Typical estimation of histidine in normal urine using photoelectric colorimeter*

(vol. of urine taken 1·5 ml./tube)

Tube	Amount of histidine HCl added (mg.)	Optical density (1 cm. cell)	*l*-Histidine HCl (mg./tube) from standard curve	*l*-Histidine HCl (mg./100 ml.) original urine
1	—	0·300	1·015	67·7
2	0·2	0·375	1·205	71·2
3	0·4	0·466	1·410	68·4
4	0·6	0·556	1·610	68·2

The effect of pH *on the production of colour*

Hunter (1922) observed a variation in the colour produced in the above reaction according to the pH of the solution, recording a dull yellow-brown in acid solution and a pink colour in alkaline solution, using NaOH for pH adjustment and a purple colour when using NH_4OH. Kapeller-Adler (1934) recommended the use of ammonia and an alkaline reaction while Langley (1941) preferred an acid reaction.

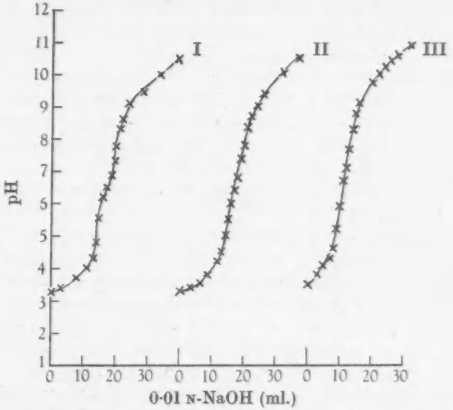


Fig. 1. Titration curves of pigments derived from histidine. Curve I. Purple pigment produced at pH 9. Curve II. Same materials as I after acidification to pH 3. Curve III. Brown pigment produced at pH 3.

Hunter's findings have been confirmed and it has been further shown that the purple pigment is formed at pH values greater than 5 and is converted to the reddish brown material by lowering the pH beyond this value. At pH values below 5 only the reddish brown pigment is produced but this is not converted to the purple substance on raising the pH. The existence of two distinct substances has been confirmed by means of titration curves determined on coloured products prepared at various pH values. For the preparation of the curves 12·3 mg. histidine HCl were dissolved in 5 ml. distilled water and then treated with excess bromine, allowed to stand for 5 min., excess bromine removed by a stream of nitrogen, the pH adjusted to the required value, the volume made to 10 ml. and the mixture heated in a boiling water bath for 5 min., cooled and diluted to 100 ml. The titration curve was determined by means of a glass electrode and pH meter. Fig. 1 shows a series of

typical curves. There is a point of inflexion in curve I at approximately pH 5·5 indicating the presence of a weak basic group, which is absent from curves II and III.

In employing this colour reaction in the routine estimation of histidine it is essential that the conditions used should be certain to yield one or other of these pigments and not a mixture of them both. Although the purple pigment is preferable for colorimetric work, the use of the brown pigment is recommended as interference by urine pigments and dissolved phosphate is considerably reduced at an acid pH. The conditions described above for the reaction yield a pH of approximately 2 and this has been found to be most satisfactory.

RESULTS

Histidine excretion by normal women

The subjects were 38 women medical students. The procedure adopted was to examine a 24 hr. urine sample at intervals of 7 days for 5 weeks in order to cover a complete menstrual cycle. The period of maximum activity of the gonadotrophic hormones is about 14 days after the onset of the period and that of minimum activity during the last 8 days before the onset of the next period (Frank, 1935). Consequently a maximum excretion of histidine would be expected 10–20 days after the onset of the period and the minimum excretion during the last 8 days of the cycle, if the suggestion of Boxer & Kapeller-Adler (1937) is correct.

The individual excretions all fell within what is recognized as the normal range, i.e. 0–400 mg./day, with two exceptions where all the samples examined had a histidine content of 400–600 mg./day. Variation in histidine content from one sample to another for the same individual was considerable and only 7 subjects showed an excretion which was constant within 100 mg. over the whole period. The maximum and minimum points of the excretions for the 38 subjects were spread evenly over the whole menstrual period, so that no correlation was apparent between histidine excretion and gonadotrophic hormone activity.

Histidine excretion in pregnancy

Two cases of pregnancy have been examined from about the third month of the pregnancy until delivery. Case I was a normal pregnancy; case II was complicated by anaemia during part of the pregnancy, although the foetus was apparently not affected and delivery was normal. As far as possible early morning urine samples were examined at weekly intervals and in the second case 24 hr. samples at monthly intervals. The early morning samples have varied in volume from 300 to 800 ml. The normal excretion of histidine of both subjects was found to be nil and the excretion of both subjects returned to this value within a few days of delivery. The blood pressure records in both cases were normal throughout the pregnancy.

The records for output of histidine in the early morning samples are shown in Fig. 2 and the 24 hr. output at monthly intervals in case II in Fig. 3.

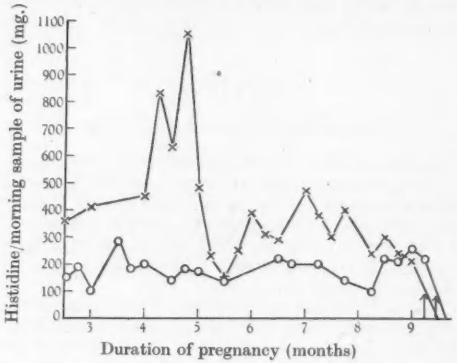


Fig. 2. Histidine excretion in two normal pregnancies. Case I, ×—×—×. Case II, o—o—o. The arrows indicate time of delivery.

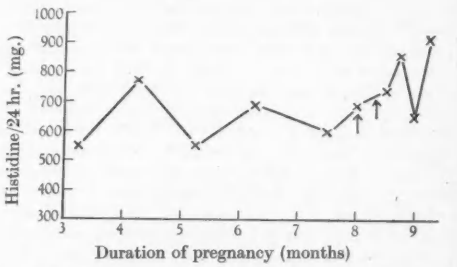


Fig. 3. 24 hr. excretion of histidine at monthly intervals in case II. Arrows indicate period of examination of diet.

There is a striking difference between the two cases; case I shows a histidine output considerably above that of case II with a definite peak at 5 months. The excretion in case II is fairly uniform but at a low level and some of the values recorded have been associated in other patients with pre-eclamptic toxaemia (Dawson, 1944).

In considering the possible causes of the increased histidine excretion in pregnancy, the increased protein intake which frequently occurs may affect the level of histidine excretion and subnormal protein intake may give rise to a lower histidine excretion. To obtain an idea of the protein intake of the two individuals concerned, their diet was weighed for 6 days and its nutritional value calculated from tables (McCance & Widdowson, 1942). The diet of case I was examined during the fifth month and case II during the eighth month of the pregnancy, this period being indicated in Fig. 3 by arrows. The results are shown in Table 2.

Table 2. *Average daily food intake over a selected period during normal pregnancy*

(Values from tables)

	Calories	Protein (g.)	Iron (mg.)
Case I	2723	92	16·4
Case II	2134	66	10·65

The protein and iron intakes are seen to be normal for case I but low for case II for a diet of late pregnancy, but despite this low protein intake there was a retention of 117 g. protein over the 6 days, calculated from urinary total nitrogen and intake.

To examine further the effect of ingested histidine on its excretion, case II ingested 2 g. of *l*-histidine hydrochloride and the 24 hr. excretion values were determined for 5 days before and 4 days after its ingestion. The period chosen was the week during which the diet was recorded and figures for histidine and total nitrogen excretion are shown in Table 3.

Table 3. *Effect of ingestion of histidine on its urinary excretion by a pregnant woman*

(2 g. *l*-histidine HCl ingested on day 6)

Day	24 hr. histidine output (mg.)	24 hr. total N output (g.)
1	690	6·6
2	702	8·4
3	685	7·3
4	693	8·2
5	378	6·8
6	784	7·5
7	665	7·5
8	470	8·2
9	314	5·3

There was a considerable drop in histidine output on the fifth control day followed by a rise after ingestion of the histidine with a steady fall over the following 3 days. Following this period the diet was improved, as shown by total nitrogen determinations on 24 hr. urine samples at weekly intervals during the last month of the pregnancy, with a figure of approximately 10·5 g. N/day and the 24 hr. histidine excretion is also seen to rise over this period (Fig. 3).

It is of interest to note that case II showed a haemoglobin level at the third month of 95%, which had dropped to 55% at the time of the dietary determination and had risen again to 92% a week before delivery. Iron salts were administered from 8½ months to delivery.

DISCUSSION

It is apparent from the above results that the histidine excretion of an individual may vary between wide limits, both in the normal and the pregnant woman. There is some evidence (Kapeller-Adler & Schiller, 1935) that dietary intake of protein which contains histidine may influence output and this may explain variations in excretion of normal individuals, but it will not explain the rise which occurs in pregnancy, although it may influence the degree of the rise. In view of the high histidine content of haemoglobin, this is a possible source of urinary histidine, since considerable quantities will be released on its breakdown and the rise in excretion in pregnancy may be associated with an increased turnover of this material. In this connexion it is interesting to note that case I above showed no abnormalities of haemoglobin level while case II had developed an anaemia by the seventh month and also showed a consistently lower histidine output than case I. In addition, during the last 6 weeks of this pregnancy there was an increased protein intake (as shown by nitrogen excretion figures) and a return of the haemoglobin level to normal accompanied by a rise in histidine output. Even if this suggested connexion between haemoglobin and urinary histidine is correct, this fact sheds no light on the causation of the greatly increased urinary histidine excretion of the normal pregnant woman.

SUMMARY

1. A colorimetric method is described for the estimation of histidine in urine.

2. In the reaction used, two pigments may be produced according to the pH at which the reaction is carried out.

3. The 24 hr. urinary histidine excretion of 38 normal women at weekly intervals during a complete menstrual cycle has been examined. No significant trends were observed and no correlation with gonadotrophic hormone activity was apparent.

4. The histidine excretion during the course of two normal pregnancies has been examined. In the first case there was a peak in the excretion at the fifth month of the pregnancy and the excretion in this case was at a generally higher level than in the second.

5. The diets of the two patients were examined; that of the second patient showed a low protein and iron intake. The possible effects of ingestion of histidine-containing protein and haemoglobin formation and breakdown on histidine excretion are discussed.

I wish to thank Prof. F. C. Happold for his continued interest and advice in this work, the patients for their willing co-operation, and Dr F. Bergel of Roche Products Ltd. for a gift of histidine hydrochloride.

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Pyruvate Oxidation in Ox Heart

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During the past decade, the *in vitro* oxidation of pyruvic acid by molecular O_2 in the presence of surviving animal tissues has been extensively studied. Published work indicates that, in general, the requirements of these pyruvate oxidation systems for maximal O_2 uptake are (i) inorganic phosphate, (ii) adenine nucleotide, (iii) a C_4-dicarboxylic acid and possibly (iv) magnesium ions (Banga, Ochoa & Peters, 1939; Smyth, 1940; Ochoa, 1943; Long, 1943, 1946).

Although it is now established that a trace of a C_4-dicarboxylic acid is needed as a catalyst for maximal O_2 uptake in the pyruvate oxidation system, the effect of the C_4-acids on the rate of pyruvate disappearance is not yet settled. Thus Annau, Banga, Blazsó, Bruckner, Laki, Straub & Szent-Györgyi (1936), working with rabbit liver, consistently found no increase in the rate of pyruvate utilization when fumarate was added, and Long (1946) obtained the same result with dialyzed pigeon brain suspensions. On the other hand, Krebs & Eggleston (1940), using pigeon breast muscle, reported an increased rate of pyruvate disappearance in one experiment and a decreased rate of disappearance in another.

The present paper summarizes the results of an investigation of the behaviour of a dialyzed ox heart preparation respiring in a medium containing pyruvate. It has been found that maximal activity requires the addition of inorganic phosphate, adenine nucleotide, fumarate and magnesium ions, and that the increased rate of O_2 consumption produced by fumarate addition is accompanied by a corresponding increase in the rate of pyruvate disappearance.

EXPERIMENTAL

Materials

Na pyruvate, Na fumarate and $MgCl_2$ were pure analytical specimens. Ba adenosine triphosphate was prepared from rabbit skeletal muscle by the method of DuBois, Albaum & Potter (1943). When required, the barium was removed as $BaSO_4$ and a stock solution prepared at a concentration of 0·01 M.

Methods

Ox heart was placed on ice as soon as possible after the death of the animal, and on arrival at the laboratory was rapidly washed and freed from fat and connective tissue. The muscle was minced finely in a Latapie mincer, the cutting blade of which was driven by an electric motor. (It has been found that the degree of subdivision of the tissue is a matter of considerable importance: thus if the mincing process is carried too far, the respiration rate of the resulting preparation is low, while if it is insufficient subsequent dialysis will fail to remove substrates and activators.)

A portion of the mince (2·8 g.) was suspended in 25·2 ml. ice-cold 0·9% KCl containing 0·04% $MgCl_2.6H_2O$ (tissue diluted 1 in 10), and several 5 ml. portions of the suspension dialyzed in collodion sacs against 4 l. of 0·4% KCl containing 0·002 M-$MgCl_2$ for 3 hr. at 2°. The dialyzer was gently rocked throughout this period so that the movement of an air bubble from one end of the sac to the other kept the tissue suspension in motion.

Portions of the dialyzed suspension, usually 2 ml., were pipetted into the manometric flasks containing substrates, activators, etc. (total volume, 3·4 ml.) and 0·2 ml. 2 N-KOH with filter paper slip was placed in the centre well. Respiration was conducted at 28°, using Barcroft manometers. After 15 min. equilibration, respiration was allowed to continue for a further 2 hr. Pyruvate was estimated by the

method of Long (1942). Respiratory quotients were determined by the direct method (Dixon, 1943) and also by the method of Dickens & Šimer (1931).

RESULTS

The effect of inorganic phosphate concentration

A typical curve is shown in Fig. 1, where the net O_2 uptake (μl./g. tissue/90 min.) is given for various concentrations of inorganic phosphate (pH 7·4) in the medium. The maximal rate of respiration is

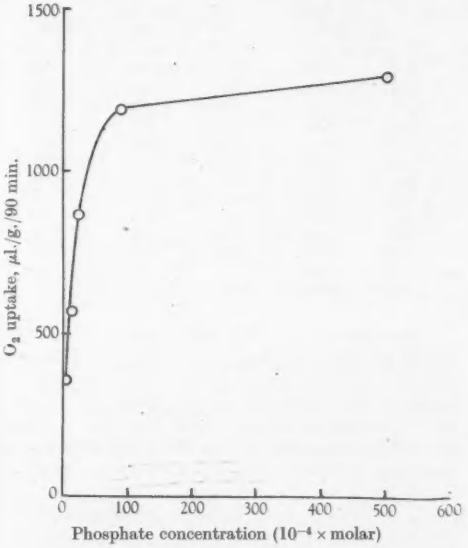

Fig. 1. The effect of inorganic phosphate concentration on the oxygen uptake by dialyzed ox heart suspension. The oxygen uptakes are net figures for 2 ml. suspension respiring in air at 28° in μl./g. wet weight for 90 min. The phosphate concentrations are expressed in molarity $\times 10^{-4}$. Each manometer contained 0·000375 M-adenosine triphosphate, 0·004 M-Na pyruvate, 0·0012 M-$MgCl_2$ and 0·0005 M-Na fumarate.

obtained at a phosphate concentration of 0·01–0·05 M. The shape of the curve indicates that the O_2 uptake would be very small, and probably zero, in the complete absence of inorganic phosphate. The residual phosphate at the end of an experiment was usually of the order of 5×10^{-4} M, and the effects of lower concentrations could not be examined. When the O_2 consumption at different phosphate concentrations is plotted against duration of respiration, it appears that the effect of inorganic phosphate is more pronounced in the later part of the experiment, i.e. that it exerts a stabilizing influence on the respiration of the tissue suspension.

It should be noted, however, that in these experiments the respiration medium was unbuffered before the addition of the inorganic phosphate, pH 7·4 being outside the range of glycine (pH 8·24–10·14) and borate (pH 7·62–9·24) buffers. As the phosphate was added in the form of a buffer of pH 7·4 it is possible that the effect produced is in part due to the stabilization of pH. This would explain the observation that the most marked effect

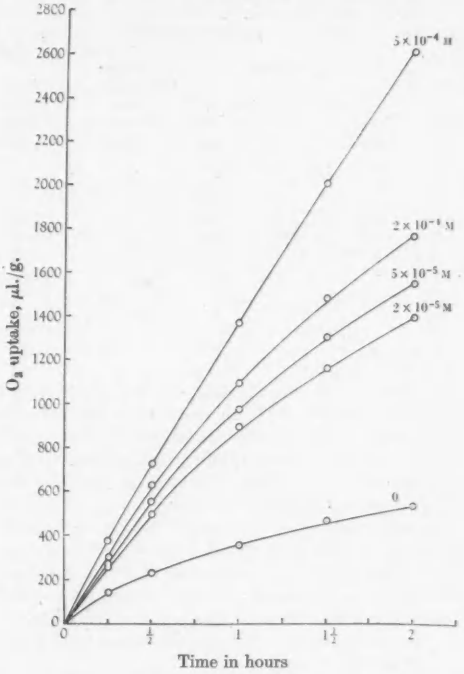

Fig. 2. The effect of adenosine triphosphate on the respiration of ox heart suspension. The oxygen uptakes are gross figures in μl./g. tissue at the concentrations of added adenosine triphosphate shown. Conditions as in Fig. 1, except that each manometer contained 0·05 M-phosphate buffer pH 7·4.

of phosphate addition was found in the later stages of the experiment, since lactic acid might be formed from residual carbohydrate during incubation.

The effect of magnesium ions

In the early part of the present work, much trouble was experienced in obtaining dialyzed suspensions of heart muscle showing a stable respiration, and many preparations were only slightly active. It was found that inactivation took place during dialysis against 0·4% KCl: storage at the

temperature of dialysis did not inactivate the suspension. The addition of Mg^{++} to the dialysis fluid was found to prevent this loss of activity. The effect produced is shown by the increase in gross O_2 uptake from 275 μl./g. tissue/hr. (av. of 12 experiments) in the absence of magnesium during dialysis, to 1220 μl./g./hr. (av. of 17 experiments) in the presence of magnesium. It is concluded, therefore, that in the absence of Mg^{++} the enzyme system is irreversibly inactivated to a marked degree. The necessity for the presence of magnesium during the oxidation of pyruvate could not, of course, be demonstrated except in experiments where dialysis had been conducted against a magnesium-free fluid: under these conditions, the addition of Mg^{++} appeared to produce some stimulation of respiration, but owing to the virtual inactivity of the preparations the results were not reproducible.

In every case an increased rate of disappearance of pyruvate has been observed following the addition of fumarate (Table 1, columns 5 and 10). In this respect, ox heart differs markedly from pigeon brain (Long, 1946).

Table 1. *Net molecular O_2/pyruvate ratios in the presence and absence of fumarate*

Each flask contained 2 ml. ox heart suspension (200 mg. wet weight of mince), 0·000375 M-adenosine triphosphate, 0·05 M-phosphate buffer, pH 7·4 and 0·0012 M-$MgCl_2$. Final concentration of added fumarate, 0·0005 M; pyruvate, 0·004 M. Total volume 3·4 ml. Respiration in air at 28°, for 2 hr.

	Fumarate absent					Fumarate present				
	O_2 uptake (μmol.)			Pyruvate disappearance (μmol.)	Net O_2/pyruvate ratio	O_2 uptake (μmol.)			Pyruvate disappearance (μmol.)	Net O_2/pyruvate ratio
Exp.	Res.	Pyr.	Net			Res.	Pyr.	Net		
1	3·1	10·4	7·3	4·1	1·78	3·9	15·5	11·6	6·3	1·85
2	—	—	—	—	—	2·0	12·0	10·0	5·8	1·72
3	4·2	6·6	2·4	3·9	0·62	10·0	20·9	10·9	8·8	1·24
4	—	—	—	—	—	5·6	15·5	9·9	7·0	1·41
5	1·6	3·1	1·5	1·0	1·50	1·9	15·4	13·5	6·1	2·22
6	5·1	7·3	2·2	3·7	0·60	8·3	19·6	11·3	7·9	1·43
7	4·8	11·4	6·6	5·2	1·27	9·1	26·4	17·3	10·3	1·68
8	2·5	2·8	0·3	2·7	0·11	6·7	12·2	5·5	4·9	1·12
9	—	—	—	—	—	3·0	14·1	11·1	6·0	1·85
10	3·4	7·5	4·1	2·7	1·52	4·8	18·0	13·2	6·7	1·97
11	—	—	—	(Undialyzed)		16·4	16·6	0·2	6·1	0·03
12	—	—	—	(Undialyzed)		19·6	22·3	2·7	8·5	0·32

The effect of adenosine triphosphate concentration

The addition of small amounts of adenosine triphosphate to the enzyme system is accompanied by a marked stimulation of the rate of respiration. The effect of increasing concentrations of this activator on the rate of O_2 consumption is shown in Fig. 2.

The effect of fumarate concentration

Catalytic amounts of fumarate (5×10^{-4} M), when added to the ox heart enzyme system respiring in pyruvate, cause a marked increase in the rate of O_2 consumption. The effect of different concentrations of Na fumarate on the rate of respiration is shown in Fig. 3. It will be seen that almost maximal activity is obtained with exceedingly low fumarate concentrations. The rate of pyruvate utilization has also been studied in the presence and absence of fumarate.

These results show that satisfactory rates of O_2 uptake and pyruvate utilization may be obtained with the following medium (final concentration in brackets): KCl (0·03 M), Na_2HPO_4-KH_2PO_4 buffer, pH 7·4 (0·05 M with respect to phosphate), $MgCl_2$ (0·0012 M), adenosine triphosphate (0·000375 M), Na fumarate (0·0005 M), and Na pyruvate (0·02 M). Under these conditions, the rate of O_2 uptake was about 1·2 ml./g. moist tissue/hr., corresponding to $Q_{O_2}^{28°}$ = approx. −7. Having regard to the difference in temperature, the activity is of the same order as that obtained by Smyth (1940) for undialyzed sheep heart suspensions respiring at 38°.

The O_2/pyruvate ratio

Simultaneous determinations of the amount of O_2 absorbed and the quantity of pyruvate disappearing were made both in the presence and absence of fumarate, and the molecular O_2/pyruvate ratio calculated. Using the normal procedure for obtaining the net O_2 uptake by subtracting the residual O_2 uptake from the total O_2 uptake in the presence of pyruvate, very divergent values for the net O_2/pyruvate ratio were found in different experiments (Table 1). It was observed, however, that the ratio varied with the extent of the residual O_2 uptake, being small when the residual uptake was large and *vice versa*. The net ratio is given by

$$\frac{O_2 \text{ (total)} - O_2 \text{ (residual)}}{\text{pyruvate disappearance}}.$$

This net ratio is the true ratio provided the residual O_2 uptake is not inhibited by pyruvate. If the residual O_2 uptake is at all inhibited in the presence of pyruvate, kO_2 (residual) rather than O_2 (residual) should be subtracted from the total O_2 uptake in the above expression, where k is the fraction of the residual uptake not inhibifed by pyruvate. If O_2 (residual) is subtracted instead of kO_2 (residual), the net ratio will always be too small, and the larger the O_2 (residual) as a fraction of O_2 (total), the smaller the net O_2/pyruvate ratio will become. This corresponds to the present case and suggests that the residual O_2 uptake is in fact inhibited in the presence of pyruvate. It was found further that a reasonably linear relationship existed between the net O_2/pyruvate ratio and the size of the residual O_2 uptake, the latter being expressed as a fraction of the total O_2 uptake in the presence of pyruvate (Fig. 4). Extrapolation of the curve to the point of intersection with the ordinate would correspond to the molecular ratio expected with a preparation which had been dialyzed so completely that the residual O_2 uptake was zero. A line fitted to the data by the method of least squares intersects the ordinate at a value of 2·505. This figure for the net ratio corresponds with the value for complete oxidation of pyruvate according to the equation

$$CH_3CO.COOH + 2\tfrac{1}{2}O_2 = 3CO_2 + 2H_2O.$$

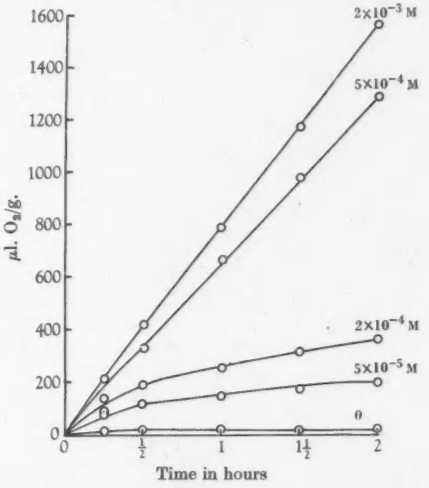

Fig. 3. The effect of fumarate on the oxygen uptake of ox heart suspension. The net oxygen uptakes are given in μl./g. tissue for the concentrations of added fumarate shown. Other conditions as for Fig. 1, except that each manometer contained 0·05 M-phosphate buffer pH 7·4.

Another inference which may be drawn from the graph is that the O_2/pyruvate ratio is not dependent on the presence or absence of fumarate, since all the points lie on the same line. This finding is in contrast to the results obtained for pigeon brain suspensions (Long, 1943, 1946), where the oxidation of pyruvate was found to be less complete in the absence of fumarate. In addition, further analysis of the graph shows that the inhibition of the residual O_2 uptake may be obtained from the slope and position of the curve. In our case, we deduce that the residual O_2 uptake is completely inhibited in the presence of pyruvate.

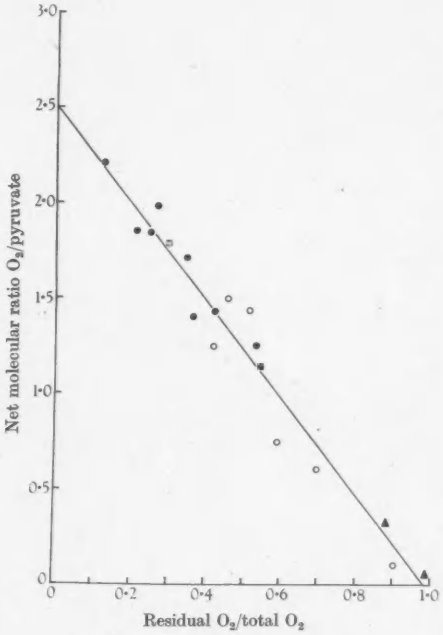

Fig. 4. The molecular ratio O_2/pyruvate. The net value for the molecular ratio O_2/pyruvate is shown plotted against the ratio O_2 residual/O_2 total. ● fumarate (0·0005 M) present; ○ fumarate absent; ▲ undialyzed heart suspension.

The respiratory quotient

The R.Q. for pyruvate oxidation in the presence of ox heart suspensions was determined by the direct method and also by the method of Dickens & Šimer (1931). The results are shown in Table 2. Since we consider that the residual respiration is completely inhibited in the presence of pyruvate, net figures have no significance, and the R.Q. in the presence of pyruvate is the only value of importance. From

Table 2. R.Q. *for pyruvate oxidation*

The R.Q. figures were obtained either by the method of Dickens & Šimer (1), or by the direct method (2). For the method of Dickens & Šimer, 1 ml. tissue suspension was used; in the direct method, 2 ml. were taken. The uptakes are in μl. O_2 and $CO_2/2$ hr. at 28°. Where both methods were used, the figures refer to 2 ml. suspension, the average value being given. Other conditions are as in Table 1. 0·0005M-fumarate was present throughout.

Residual			Pyruvate present			
O_2 uptake (μl.)	CO_2 output (μl.)	R.Q.	O_2 uptake (μl.)	CO_2 output (μl.)	R.Q.	Method
76·8	74·7	0·97	255·0	266·0	1·04	(1)
22·8	19·9	0·87	97·3	114·6	1·18	(1)
165·0	139·0	0·84	697·0	789·0	1·13	(1), (2)
88·2	89·0	1·01	221·5	267·0	1·21	(1)
24·6	20·6	0·84	193·0	219·5	1·14	(1)
77·9	76·3	0·98	381·6	457·0	1·20	(1), (2)
64·0	48·7	0·76	412·0	483·0	1·17	(1), (2)
	Average	0·88		Average	1·15	

Table 2, the R.Q. is seen to be of the order 1·2. This also agrees with the calculated value for complete oxidation.

DISCUSSION

The results quoted in the experimental part of this paper lend themselves to a comparison between the pyruvate oxidation processes in ox heart and in other tissues. The tissue which has been studied most intensively under comparable conditions is finely divided pigeon brain suspension, which after dialysis requires inorganic phosphate, adenine nucleotide and a C_4-dicarboxylic acid for maximal rate of respiration and pyruvate utilization (Banga *et al.* 1939; Long, 1943, 1946).

There would appear to be some difference between these two tissues in their requirement for magnesium, since pigeon brain is not inactivated by dialysis in the absence of Mg^{++}. The addition of Mg^{++} after dialysis has not been found to affect the respiration of pigeon brain suspensions (Long, unpublished), although Ochoa (1939) has claimed that Mg^{++} is necessary in experiments carried out under similar conditions. It would seem therefore, either that Mg^{++} is not necessary for pigeon brain, or that it is more firmly bound to the enzyme than in heart.

Heart and brain preparations also differ in the effects produced by the C_4-dicarboxylic acids on the course of pyruvate oxidation in these tissues. Most animal tissues, respiring in a medium containing pyruvate, respond to the addition of fumarate, the O_2 uptake being generally at least doubled. Heart and brain behave similarly in this respect. They differ, however, in the effect produced by fumarate on the rate of pyruvate utilization. Thus in brain tissue, the extent of pyruvate disappearance is not increased by fumarate addition, the effect of which is to bring about complete rather than partial oxidation of that amount of pyruvate which is utilized in the absence of fumarate (Long, 1946). In heart, on the other hand, fumarate addition increases the actual rate of pyruvate disappearance. It does not affect the completeness of the pyruvate oxidation, since even without added fumarate complete oxidation takes place. It would appear, therefore, that animal tissues differ in this respect, heart and possibly pigeon breast muscle (Krebs & Eggleston, 1940) contrasting with brain (Long, 1946) and liver (Annau *et al.* 1936).

The method adopted in the present work for determining the true O_2/pyruvate ratio is of some general interest, since it may be applied to the study of any tissue in which the residual O_2 uptake of different preparations varies over a fairly wide range. It is clear that if in our experiments the normal method of subtracting the residual from the total O_2 uptake had been followed, it would have been wrongly concluded that pyruvate was incompletely oxidized by all our preparations, and that the addition of fumarate led to a relatively more complete oxidation of pyruvate. The residual respiration of heart muscle may be due to the presence of reserve carbohydrate in a non-dialyzable form which is broken down during the respiration period and oxidized as pyruvate. The addition of pyruvate in large amounts would almost certainly inhibit the formation of pyruvate from residual carbohydrate, and simple addition of the residual O_2 consumption to that due to pyruvate would not be expected to occur. It would seem that in work of this kind it is advisable to examine the data carefully before using net values calculated by subtracting the residual from the total O_2 uptake. A reinvestigation of the results for pigeon brain (Long, 1943, 1946) has indicated that the normal procedure of subtracting the residual O_2 uptake was a valid procedure in that particular case, since the same values for the O_2/pyruvate ratio were obtained when the method used for the heart preparations was employed. It should be pointed out, however, that the latter method is difficult to apply in the case of brain because of the relative constancy of the residual O_2 uptake. The

method used in calculating the results for the heart preparations, though offering uncertainties of its own, is probably nevertheless the more general and desirable procedure.

SUMMARY

1. The greatest activity of the pyruvate oxidation system by finely divided dialyzed ox heart suspensions is observed in the presence of inorganic phosphate, adenine nucleotide and fumarate added simultaneously.

2. The presence of Mg^{++} is necessary to prevent irreversible inactivation of the enzyme during dialysis.

3. Addition of catalytic amounts of fumarate (0·0005 M) greatly increases both the O_2 uptake and pyruvate disappearance.

4. The pyruvate is completely oxidized to CO_2 and water, the molecular ratio O_2/pyruvate deduced by extrapolation from the experimental data being 2·5 and the R.Q. 1·2.

5. A comparison is made between the course of pyruvate oxidation in ox heart and pigeon brain suspensions.

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Haemolysis and Fixation of Erythrocytes by X-Rays

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It has previously been reported (Halberstaedter & Goldhaber, 1943; Halberstaedter, 1944) that following irradiation with an X-ray dose of 2×10^6 r. at high intensity, erythrocytes *in vitro* undergo either haemolysis or fixation. The type of the response depends on the dose of X-rays, on the concentration of the erythrocyte suspension and on the medium in which they are suspended during irradiation. At high cell concentrations (>0·5%) the effect of the irradiation is always haemolysis independently of the nature of the medium. At low concentrations (<0·5%), on the other hand, the type of the response obtained depends on the medium in which the cells are suspended. At low concentration, the erythrocytes are resistant to haemolysis if irradiated in a medium of 0·9% sodium chloride solution, but undergo complete haemolysis if irradiated in a medium of isotonic glucose or 1 vol. serum : 3 vol. of 0·9% sodium chloride solution.

Continuing this investigation, the influence of different media on the response of erythrocytes to X-ray irradiation has been studied. Experiments were made to determine what serum constituent is responsible for the haemolytic reaction of erythrocytes which are irradiated at low concentration with an X-ray dose of 2×10^6 r.

EXPERIMENTAL

Blood. Fresh rabbit or human blood was defibrinated, the erythrocytes washed twice with 0·9% NaCl and suspended in concentrations of 0·1% and 1% (v/v) in the different media. Small hanging drops under mica slips were irradiated and the effects observed with the microscope.

X-rays. Irradiation was carried out by means of a demountable X-ray tube with copper anode at a tension of 35 kV. and a current of 40 ma. The window of aluminium foil was 30μ, and the mica slip for the hanging drop 0·02–0·025 mm. in thickness. The X-ray intensity at the distance of the hanging drop (3·5 cm.) was about 95,000 r./min. The dose was always the same, namely 2×10^6 r.

Irradiation media. The effect of irradiation under the above-mentioned circumstances on 0·1% erythrocyte suspensions in saline (0·9% NaCl solution) was always fixation ('saline effect', S.E.), but in a medium of 1 vol.

serum : 3 vol. of saline complete haemolysis always took place. Therefore 0·9% NaCl as well as a mixture of 3 vol. 0·9% NaCl : 1 vol. serum were employed as standard control media. For the experiments, different isotonic (i.e. isosmotic with normal saline) salt solutions mixed with 3 vol. saline were used as media. Graded dilutions of these mixtures as well as of the standard serum-saline were prepared for comparison. Comparisons were also made in some cases between the effect of the isotonic salt solutions as such and that of the undiluted serum. The pH was controlled by potentiometric measurements.

All experiments were carried out at room temperature (19–25°), and the controls were always observed simultaneously with the experiments and under the same conditions.

RESULTS

Range of saline effect

Isotonic solutions of many alkali and alkali-earth salts ($CaCl_2$, KCl, $MgSO_4$) resemble saline in their effect on the X-ray response of erythrocytes. Isotonic $NaNO_3$ and $(NH_4)_2SO_4$ likewise produce the S.E. but diverge slightly from the behaviour of the other salts in some other respects. In $NaNO_3$ solution the X-ray haemolysis of 1% erythrocyte suspension proceeds much more slowly than in NaCl and the haemolysis is not complete. In $(NH_4)_2SO_4$ solutions spontaneous haemolysis of non-irradiated 0·1% suspensions occurs. The failure of haemolysis in that medium after irradiation shows that the effect of X-rays in this case is in fact a kind of fixation.

Definite evidence of the state of fixation, i.e. of the total inability to become haemolyzed, of erythrocytes irradiated at 0·1% cell concentration in any of the above salt media was obtained from experiments with saponin and eosin. Saponin, a powerful haemolytic agent, produced immediate haemolysis of non-irradiated suspensions of erythrocytes at 0·1% concentration but failed to dissolve the same suspensions after they were irradiated with 2×10^6 r. *in vitro*. The irradiated cells absorbed eosin readily in the manner typical of fixed cell preparations.

Among non-electrolytes investigated, glycerol in isotonic solution diluted with 3 vol. of isotonic NaCl produced S.E. Considering the chemical relationship of glycerol and glucose this finding is somewhat surprising.

A totally different type of influence is exerted by irradiation in isotonic solutions of alkali phosphates, alkali bicarbonates, glycine and urea. In both KH_2PO_4 (pH 4·5) and Na_2HPO_4 (pH 9·1) as well as in a mixture of the two at the physiological pH (7·3),

Table 1. *Effect of X-ray irradiation* (2×10^6 r.) *on erythrocytes in different media with and without addition of serum*

(Media: 1 vol. isotonic salt solution + 3 vol. 0·9% NaCl. Concentration of erythrocytes 0·1 and 1·0%.)

	Erythrocytes irradiated						Erythrocytes non-irradiated	
	Media (medium + saline)			Media + ¼ vol. serum				
Medium	pH	(0·1%)	(1·0%)	(0·1%)	(1·0%)	pH	(0·1%)	(1·0%)
NaCl	6·95	0 +Sap. 0	*c*	*c*	*c*	7·35	0	0
KCl	6·95	0 +Sap. 0	*c*	*c*	*c*	7·35	0	0
$CaCl_2$	6·9	0 +Sap. 0	*c*	*c*	*c*	7·25	0	0
$(NH_4)_2SO_4$	6·9	0 +Sap. 0	Not *c* +Sap. *c*	*c*	*c*	7·3	*c*	0
$MgSO_4$	6·9	0 +Sap. 0	24 hr. 0 +Sap. *c*	*c*	*c*	7·25	0	0
$NaNO_3$	6·95	0 +Sap. 0	Fading +Sap. not *c*	Fading	*c*	7·35	0	0
Glycerol	6·9	0 +Sap. 0	*c*	*c*	*c*	7·3	0	0
Glucose	6·9	*c*	*c*	—	—	7·3	0	0
KH_2PO_4	4·5	*c*	*c*	—	—	—	0	0
Na_2HPO_4	9·1	*c*	*c*	—	—	—	0	0
Phosphate mixture	7·3	*c*	*c*	—	—	—	0	0
Glycine	—	*c*	*c*	—	—	—	0	0
Bicarbonate	7·35	*c*	*c*	—	—	—	0	0

0 = No haemolysis, *c* = complete haemolysis, Sap. = saponin solution.

Saponin added to the non-irradiated controls always gives complete haemolysis.

suspensions of erythrocytes at all cell concentrations were susceptible to X-ray haemolysis exactly as in a medium of isotonic glucose or serum-saline mixture. Glycine, like serum or phosphate, prevented fixation. Isotonic urea solution caused haemolysis of erythrocytes, and therefore experiments on the effect of X-rays in this medium could not be performed. Erythrocytes also underwent haemolysis when suspended in isotonic bicarbonate; no haemolysis occurred, however, when a mixture of 1 vol. isotonic bicarbonate and 3 vol. saline served as medium. The response of the erythrocytes in the latter case resembled their behaviour in either serum, glucose, phosphate or glycine medium.

Among the substances which have been discussed, three—glucose, phosphate and bicarbonate—are constant and physiologically active components of normal serum. Their proportions/100 mol. NaCl are, approximately, 5 mol. glucose, 1 mol. phosphate and about 20 mol. bicarbonate (the amount of the latter depending on the varying CO_2 saturation of arterial and venous blood). The effect of separate addition of each of these three substances on the response of erythrocytes in saline to X-ray irradiation was studied.

Addition of glucose to saline up to the normal blood sugar concentration did not prevent S.E. Phosphate prevented S.E. only when added in overwhelming excess. In mixtures of phosphate and NaCl containing less than 80 % (v/v) phosphate, S.E. persisted.

Table 2. *Rabbit erythrocytes* (0·1 *and* 1·0 % *suspensions*) *irradiated with* 2×10^6 r. *in phosphate solution*

(30·5 ml. 2·11 % (M/6·4) KH_2PO_4 + 69·5 ml. 1·84 % (M/9·6) $Na_2HPO_4.2H_2O$ (isotonic) diluted with saline, pH 7·3.)

Vol. concentration of phosphate solution in saline (%)	Erythrocyte concentration (0·1 %) Haemolysis	Erythrocyte concentration (1 %) Haemolysis
0	None	Complete
20	None	Complete
40	None	Complete
60	None	Complete
80	Marked	Complete
100	Complete	Complete

Bicarbonate, on the other hand, prevented S.E. even when minute quantities of it were added to saline.

Table 3. *Human erythrocytes* (0·1 % *suspensions*) *irradiated with* 2×10^6 r.

(Medium: different dilutions in saline of an isotonic mixture of 2·5 ml. $NaHCO_3$ and 97·5 ml. saline.)

Dilutions	
1 : 3	Immediate complete haemolysis
1 : 10	Complete after 40 min.
1 : 20	Marked haemolysis
1 : 50	Slight haemolysis
1 : 100	No haemolysis

Identification of the S.E. *antagonist of serum*

Both the dialyzable constituents of serum and the serum protein (freed from salts other than NaCl by dialysis against isotonic saline) were tested.

In a mixture of 100 vol. NaCl, 2 vol. KCl, 2 vol. $CaCl_2$, 1 vol. phosphate, 2 vol. glucose and 10 vol. $NaHCO_3$ (all isotonic), erythrocytes in a suspension of 0·1 % cell concentration behaved under irradiation exactly as in serum (haemolysis). Omission from this mixture separately or jointly of $CaCl_2$, KCl, PO_4 and glucose did not affect the response obtained. On the other hand, omission of bicarbonate from the mixture caused the appearance of S.E. (fixation) both in absence or presence of $CaCl_2$, KCl, PO_4, or glucose.

Serum was dialyzed for 24–48 hr. in an ice-box against a large excess of repeatedly renewed saline through a cellophan membrane. At the end of this time micro-determinations for sugars were invariably negative, and the dialyzed serum was presumed to contain practically no dialyzable constituent except NaCl. During dialysis, the surface of the external solution was kept carefully below the surface of the internal one, the hydrostatic pressure preventing entry of water and even causing some loss of water through filtration. After dialysis the vol. of the serum could be made up to the original vol. with saline.

The dialyzed serum showed the S.E. (fixation) and its behaviour in this respect was indistinguishable from that of protein-free saline solution. In dialyzed serum to which bicarbonate was added in an amount barely corresponding to the minimum

Table 4. *Influence of undialyzed and dialyzed serum with or without addition of* $NaHCO_3$ *on the effect of X-rays on erythrocytes*

(0·1 % suspensions of human erythrocytes in different dilutions of undialyzed serum, dialyzed serum, and dialyzed serum plus 2·5 % of its vol. of isotonic $NaHCO_3$, irradiated *in vitro* with 2×10^6 r.)

Dilution in saline	Undialyzed serum	Dialyzed serum	Dialyzed serum plus $NaHCO_3$
1 : 4	Complete haemolysis	No haemolysis	—
1 : 10	Complete haemolysis	No haemolysis	Very strong haemolysis
1 : 25	Almost complete haemolysis	No haemolysis	Very strong haemolysis
1 : 50	No haemolysis	No haemolysis	No haemolysis
1 : 75	No haemolysis	No haemolysis	No haemolysis

occurring in normal serum (20 % v/v of an isotonic $NaHCO_3$ solution), the erythrocytes were haemolyzed by X-rays at all cell concentrations. Addition to dialyzed serum of bicarbonate alone thus regenerates the original quality of serum as an irradiation medium.

For quantitative evaluation of the anti-S.E. potency of genuine and of bicarbonate-reconstituted dialyzed serum respectively, the two media were added in varying concentrations to saline and the dilution in saline to which the anti-S.E. persisted was then determined.

As Table 4 shows, haemolysis does not occur in dialyzed serum. Undialyzed serum and dialyzed serum reconstituted with sodium bicarbonate are similar in their behaviour as media at all dilutions with saline. Haemolysis still occurs in both cases at a dilution of 1 : 25, but at 1 : 50 fixation takes place.

DISCUSSION

The experiments presented show that X-rays can act on erythrocytes in two opposing and mutually excluding ways: (*a*) haemolysis. and (*b*) fixation. The particular effect produced by a given X-ray irradiation, i.e. the alternative which takes precedence and excludes the other, depends on three factors: intensity and dose of the irradiation, the cell concentration of the erythrocyte suspension, and the nature of the medium. The three factors are interdependent. The dependence of the nature of the response (haemolysis or fixation) on the cell concentration exists only in certain media (saline type), while in others (serum type) haemolysis is produced by an effective X-ray dose independently of the cell concentration. The dependence of the nature of the response on the composition of the medium exists only at low cell concentrations ($< 0{\cdot}5\,\%$), whereas at high cell concentrations haemolysis is induced by an effective dose independently of the composition of the medium.

The conditions determining haemolysis and fixation following irradiation of erythrocytes with X-rays are thus quite different. Fixation only occurs if three conditions are fulfilled: (1) the cell concentration of the erythrocyte suspension is less than $0{\cdot}5\,\%$; (2) the X-ray dose is 2×10^6 r. or more; (3) the medium is of the saline type. On the other hand, haemolysis occurs provided that only one of the following conditions is fulfilled: the erythrocyte concentration is $0{\cdot}5\,\%$ or more, the X-ray dose is smaller than 2×10^6 r., or the medium is of the serum type. Haemolysis is therefore the ordinary, i.e. the most general, response to X-ray influence, and fixation is only produced in exceptional circumstances. The following further important difference should also be noted: the haemolysis elicited by irradiation can continue for many hours or days, the time required for complete haemolysis being dependent on the dose employed. In fixation, however, the complete effect is produced within the time of the irradiation. When at the completion of the irradiation fixation has not occurred the response later obtained is always haemolysis.

Haemolysis caused by X-rays is considered by Ting & Zirkle (1940) and by Liechti & Wilbrandt (1941) to be an osmotic effect. According to Ponder (1936) and Davson (1943) haemolysis is not a simple permeability process. The escape of haemoglobin 'results from the formation of holes in the membrane, which are large compared with the haemoglobin molecule, so that the escape is probably determined by simple laws of diffusion in an aqueous medium'. The membrane alterations which occur in X-ray haemolysis can probably be attributed to a direct action of the X-rays on the membrane. The known facts do not in any case contradict the assumption that the mechanism of the action is the production within the membrane of ionization centres which bring about an alteration whereby the escape of haemoglobin becomes possible.

On the other hand, we have to admit that the interpretation of the mechanism of the fixation of erythrocytes by X-rays (S.E.) is obscure. S.E. is produced by X-rays in many neutral salt solutions, but cannot be characterized as an electrolyte effect since it is also produced in glycerol and since it is opposed by specific electrolytes, like phosphate and bicarbonate, as well as by glucose and glycine. The explanation in terms of pH is ruled out by the fact that primary and secondary phosphates in a wide range of pH values (4·5–9·0) show no difference in behaviour and that bicarbonate and phosphate of the same pH behave quite differently.

The experimental findings with regard to fixation of erythrocytes by X-rays cannot be explained on the assumption that the effect of the irradiation in this case is direct. Since fixation must be brought to completion during the irradiation itself it is comprehensible that a much larger dose is required for fixation than for haemolysis. If fixation were brought about by the direct action of the X-rays, i.e. on the basis of the direct hit mechanism, it would be incomprehensible that it depends at a given dose and in a given medium (saline type) on the erythrocyte concentration. The observation that fixation following irradiation with doses greater than 2×10^6 r. is prevented by the presence in the medium of serum and certain other substances is likewise difficult to reconcile with the view that the effect of the X-rays on the erythrocytes is direct.

The theory of indirect action through 'activation' of accompanying substances, on the other hand, seems *prima facie* in good accord with the observed dependence of the irradiation effect on the composition of the irradiation medium. But it suggests no ready explanation for the effect of the cell concentration. It might be argued that a regular dependence of radiation effect on substrate concentration is to be expected on the basis of the indirect action theory, as the ratio: substrate concentration (number of cells)/density of 'activation centres' for a given radiation intensity and a given medium naturally depends on the substrate concentration. In our case, however, the change of the nature of response to irradiation with increasing cell dilution is not gradual, but assumes the form of an appearance of a qualitatively different effect (S.E.) at

some specific cell concentration level (<0·5%). Moreover, 'activation centres' of 'indirect action' theories have mostly been regarded as 'activated water molecules' involving production of H_2O_2 and/or H^+ (Fricke, 1934, 1935). In our experiments there is nothing which suggests oxidation as an explanation for the different behaviour of media of saline and serum type, as both groups are not defined by different oxido-reduction properties. The irrelevance of an H^+ effect has already been pointed out.

The influence of the concentration and of the nature of the medium in our experiments on erythrocytes has an analogy in results obtained with other objects. Dale (1940) first demonstrated the dependence of the X-ray effect on enzymes on the substrate concentration and the medium. Similar results have been reported for virus by Lacassagne & Nyka (1938), Friedewald & Anderson (1941), Luria & Exner (1941). Evans, Slaughter, Little & Failla (1942) showed that the effect of X-ray irradiation on *Arbacia* sperm depends on the medium and on the concentration of the irradiated sperm suspension. A growing susceptibility to irradiation with diminishing concentration of the irradiation substrate has always been described. Dale (1943) and other investigators consider these phenomena to be indirect effects mediated by ionization of the water molecules.

In the case of the effect of ultra-violet irradiation on taka-carbohydrases it has been shown (Goldhaber & Leibowitz, 1943) that the alleged dependence of the inactivation effect on the concentration of the target is really a dependence not on the concentration of the active enzyme molecules as such but rather a dependence on the concentration of substances (proteins?) which accompany the enzyme, but which do not participate in its activities. The same applies also in the case of certain proteolytic enzymes of pancreatin (Goldhaber, Leibowitz & Feigenbaum, unpublished results). In the case of living cells, however, it is clearly hazardous to venture any statement on what constituents are active in the mechanisms of haemolysis and fixation and what are merely accompanying substances.

Haemolysis and fixation have been observed in experiments with photodynamic substances on erythrocytes. Blum (1930) found that haemolysis and fixation are brought about by non-irradiated eosin at defined hydrogen ion and dye concentrations. Irradiation shifts the region of occurrence of these phenomena into lower concentrations of the dye. Blum (1935) also found that H_2O_2 and eosin may reinforce each other in producing haemolysis and fixation. In our experiments no dependence on hydrogen ion concentration or on oxidation processes was observed.

SUMMARY

1. Suspensions of erythrocytes in a concentration range from 5% (v/v) down to about 0·5% respond to irradiation with an effective X-ray dose by haemolysis independently of the nature of the medium in which they are suspended.

2. At cell concentrations below 0·5%, the response of erythrocytes to irradiation depends on the medium in which they are suspended: (*a*) In isotonic glucose, KH_2PO_4, Na_2HPO_4, $NaHCO_3$, and glycine the response to an effective X-ray dose is haemolysis. (*b*) In isotonic NaCl, KCl, $CaCl_2$, $NaNO_3$, $(NH_4)_2SO_4$, $MgSO_4$, and glycerol the effect of an X-ray dose of 2×10^6 r. and more is not haemolysis but cell fixation, the total loss of the ability to haemolyze.

3. Addition to media listed in 2 (*b*) of serum, phosphate or bicarbonate prevents fixation. The fixation-opposing activity of phosphate is considerably below that of serum or bicarbonate. The bicarbonate content of serum accounts completely for its high activity in preventing fixation.

4. The relevance of the nature of the medium and of the cell concentration of the erythrocyte suspension for the occurrence of the fixation effect points to some 'indirect action' mechanism of the irradiation.

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The Amino-aciduria in Fanconi Syndrome. A Study Making Extensive Use of Techniques Based on Paper Partition Chromatography

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It has long been known, and confirmed by direct isolation experiments (Honda, 1922, 1923; Wada 1930), that small quantities of many amino-acids are normally excreted in the urine, but that their excretion in large amounts occurs only in rare pathological conditions. The term amino-aciduria is reserved for such cases. It was first described in acute yellow atrophy of the liver, where its onset coincides with a large rise in the blood level of amino nitrogen and is presumably due to a simple overflow from the blood into the urine.

The reported occurrence of amino-aciduria in the Fanconi syndrome (Fanconi, 1936; McCune, Mason & Clarke, 1943) is of distinct interest because in this syndrome any possible relationship to acute yellow atrophy can be excluded. The mechanism of the amino-aciduria was therefore likely to be entirely different, and although it was suggested that a low kidney threshold for amino-acids may be the causative factor, no systematic work was carried out. The blood level of amino nitrogen was not determined and moreover the earlier workers seem to have thought that the amino-acids in the urine could be titrated quantitatively by the method for organic acids of Van Slyke & Palmer (1920).

The literature concerning the syndrome is surveyed by McCune *et al.* (1943). The syndrome is of unknown etiology and usually presents as typical rickets in a child of 2–5 years, although cases in adults presenting as osteomalacia have also been reported. The bony lesions are not cured by normal doses of vitamin D. In addition the patients have renal glycosuria and the constituents of the blood show changes which clearly distinguish the disease from renal rickets (renal hyperparathyroidism), namely, low inorganic phosphate and a normal blood urea and non-protein nitrogen. The alkaline phosphatase is usually raised and the alkali reserve reduced. The urine has a high ammonia coefficient. Cirrhosis of the liver and generalized cystinosis may be found at post-mortem. The close resemblance to the syndrome described by Milkman (1930, 1934) should be noted.

It was thought profitable to study further the amino-aciduria in Fanconi syndrome. The introduction by Consden, Gordon & Martin (1944) of a comparatively simple technique for the detection of amino-acids has provided a most effective weapon for the purpose. It has already been shown to be applicable to pathological specimens of urine and other body fluids (Dent, 1946*a*). The chronic loss of amino-acids in the urine might be the cause of some of the symptoms, or if it were merely the result of a low renal threshold in the presence of normal amino-acid metabolism then such a patient would enable information to be obtained with comparative simplicity as to the normal amino-acids present in blood under various conditions. At the onset of the investigation certain specific points were set out for study:

(1) What is the nature and extent of the amino-aciduria?

(2) What is the mechanism of excretion?

(3) Is there any relation between the amino-aciduria and the renal glycosuria?

(4) Is the amino-aciduria the primary cause of the disease?

(5) Is there any disturbance in sulphur metabolism such as might account for the cirrhosis of the liver or the cystinosis?

Except for (4), these questions have been answered fairly satisfactorily. A preliminary report of some of the results described here has already appeared (Dent, 1946*b*).

EXPERIMENTAL

Patients studied

Full clinical details of the patients E.C., V.R. and E.B. will be reported elsewhere (Stowers & Dent, 1947; Hunter, 1947). All showed the typical features of the disease. E.C. and V.R. who came to post-mortem showed marked cirrhosis of the liver with recent primary carcinoma. V.R. in addition had multiple metastases in the lungs and many other organs. E.C. had had repeated haematemeses from oesophageal varices and died in liver coma. E.B. has signs of increasing portal obstruction but is otherwise fairly well.

Patient E.C. This patient (age 34) has provided nearly all the material for this study. Three 24 hr. specimens of urine had been collected from him

during the previous Oct., Nov. and early Jan. when he was attending as an outpatient. He was given, on admission in late Jan. a high protein diet with extra milk. However, frequent attacks of vomiting interfered drastically with his intake. It was only possible to estimate his daily protein intake for one week in Jan. Urines were collected from midnight to midnight by the nursing staff till 4 Feb. 1946, when the more reliable method of collecting the urine directly by means of a funnel connected to a Winchester quart bottle under the bed was instituted. Thymol was used as a preservative till 25 Jan. 1946 when the frequent occurrence of bacterial infection led to 50 ml. of approx. 1·5 N-HCl being used. The acid was placed in the bottle and the urine added as passed. The completed specimens were kept at 4°. According to Van Slyke, MacFadyen & Hamilton (1943), no loss of amino nitrogen occurs during 5 months at 4° with only thymol as a preservative.

The amino-acids were given by mouth, suitably flavoured and suspended or dissolved in water, at 4 a.m. after the patient had emptied his bladder. The urine was then collected 3 and 6 hr. afterwards. The rest of the day's urine was collected separately with the small amount, if any, of the urine voided at 4 a.m. added to it.

Patient V.R. The urine that could be collected from this patient during the last 48 hr. was added as passed to 100 ml. approx. 1·5 N-HCl in the stock bottle. Owing to the small volume passed (730 ml.) the final acid concentration was much higher than originally intended. For various reasons the urine was allowed to stand at room temperature for a considerable time. The amino nitrogen was determined a few days after, but the chromatogram was not done till a month later.

Patient E.B. Four samples of urine passed on 7, 10, 11 and 13 Mar. 1946 were obtained. None contained albumin or bilirubin; all contained normal traces of cystine and urobilin. Reducing substances (Benedict's reagent) were found only in the urine of 13 March and this was the only urine containing an excess of amino-acids detectable by the one-dimensional chromatogram. The further investigations were therefore confined to this specimen of urine.

Methods

Chemical determinations

Amino nitrogen was determined in the urines by the formol titration method (Van Slyke & Kirk, 1933). The vacuum distillation with baryta was carried out for 30 min. and continued longer if the distillate was still alkaline, as it often was.

Amino nitrogen was determined (by Miss E. Richardson) in the serum by the ninhydrin CO_2 method.

Nitrogen was determined throughout by the micro-Kjeldahl method.

Inorganic and ethereal sulphate were determined by the standard gravimetric methods (Peters & Van Slyke, 1932), and neutral sulphur by Benedict's method, except that after 2 Feb. 1946, nitric and perchloric acids were used as the oxidizing mixture (Pirie, 1932), because this is more reliable for determining all the sulphur in methionine.

Methionine was determined by the hydrogen peroxide oxidation method of Toennies & Callan (1939) as applied to urine by Albanese, Frankston & Irby (1944).

Cystine was regularly tested for by the qualitative cyanide-nitroprusside reaction (Brand, Harris & Biloon, 1930). Usually the test gave faint colours; occasionally definitely stronger than normal but never in any way comparable to the reaction obtained from known cystinurics. No quantitative methods were therefore applied.

Calcium was also tested for qualitatively by Sulkowitch's reagent. No excessive outputs were detected.

Inorganic phosphate was determined by uranium titration (Peters & Van Slyke, 1932) using Tinct. Cocci as internal indicator.

Glucose was determined by Benedict's volumetric method.

Partition chromatograms for detecting amino-acids

The method of Consden *et al.* (1944) as applied to urines by Dent (1946*a*) was used throughout, 25 μl. of urine being taken each time. Phenol was always used as the first solvent, 1 ml. of conc. ammonia (sp.gr. 0·880) being added to the cabinet. Collidine was used as the second solvent. The leading edge of the phenol always carried forward a great deal of dirty brown material, partly from the paper, partly presumably from decomposition of the phenol. This was always cut off with scissors before running the collidine in the other direction, as otherwise it interfered with the flow of the collidine. The strength of the ninhydrin colours was compared against an arbitrary colour standard, as soon as developed, owing to the marked fading which occurred in a few days. The scale used was divided into 10 units, 1 being a very faint colour, 10 a deep purple. The spots of rather different shade could only be roughly compared.

The identification of ninhydrin reacting substances on the chromatograms was attempted in the first place by comparing the positions of the various spots (R_F values) against a synthetic mixture of pure amino-acids run on another chromatogram simultaneously in the same cabinet, very good agreement being usually obtained (Dent, 1946*a*). In more doubtful cases the pure amino-acids were added to the urine and their superposition with the unknown on the developed chromatogram confirmed or otherwise. This was always necessary if only a few spots appeared, as in normal urines. The stability of the spots after hydrolysis of the urine had also to be confirmed. Confusion with a peptide is the only serious source of error, there being an almost infinite number of possible peptides while the number of pure amino-acids, i.e. spots persisting after hydrolysis, is strictly limited. The R_F values found here differ slightly from those reported by Consden *et al.* (1944), and are reported here together with many new values in Table 6.

The above methods were applied to the identification of the following amino-acids in the urine of E.C.: cysteic acid, aspartic acid, glutamic acid, serine, glycine, threonine, alanine, α-amino-*n*-butyric acid, histidine, citrulline, arginine, valine, leucine (and/or isoleucine), phenylalanine, tyrosine, proline, hydroxyproline. Unfortunately the

technique as here described does not distinguish between eucine and *iso*leucine, and further investigation is necessary before it is known which of these is present. Methionine travels to a position so close to that of the leucines that a confusion is possible unless both are present in sufficient strength as to be separately visible, as in the urines of E.C. after methionine feeding. The identification of these amino-acids was confirmed by treating the urine on the paper, before starting the chromatogram, with 10μl. of 30% H_2O_2 (perhydrol) which was then allowed to dry. This converted the methionine quantitatively into the sulphone which then appeared in a much less ambiguous position near valine. The leucines were unchanged and remained in their original position. The validity of this procedure was readily confirmed by using synthetic mixtures and by treating similarly the urine of E.C. after methionine feeding. The H_2O_2 treatment also served to show cystine, which it quantitatively converted into cysteic acid. Cystine usually decomposed completely during the chromatography; cysteic acid however is quite stable once formed. Possibly the H_2O_2 pre-treatment will eventually become the standard method for showing up on the one chromatogram all the amino-acids present in a mixture. Its use was avoided here in routine testing owing to the doubt as to the possible reactivity of H_2O_2 under these conditions with other amino-acids, especially with tryptophane (cf. Toennies & Homiller, 1942). Unfortunately, no α-amino-*iso*butyric acid was at hand for comparison; it is believed however that it would be distinguishable from the *n*-butyric derivative. The matching with the urine was done particularly carefully in this case.

The presence in the urine of histidine and tyrosine, both of which give weak reactions with ninhydrin, was readily confirmed in the present study by means of the one dimensional chromatogram run with collidine. Under these conditions histidine has an R_F value of 0·10–0·14 and tyrosine of 0·44. They are thus well separated on the paper strip and are readily developed by stroking the paper first with diazotized sulphanilic acid and then with sodium carbonate solution (Pauly, 1904) each from a small micro-pipette. It makes no difference if the strip has first been treated with ninhydrin. The diazo reaction is more sensitive than the ninhydrin method for these two amino-acids, histidine gives a bright red colour with 5μg. and tyrosine a golden brown with 20μg. (after running on the collidine strip). Normal urines show only a trace of histidine and no tyrosine; the urines of E.C. gave a much stronger histidine colour and a definite reaction for tyrosine. When a normal urine is concentrated to one-tenth vol. *in vacuo* it usually shows tyrosine by this method as well as another substance reacting to give a chocolate brown colour with the Pauly reagent, and having an R_F value of 0·64. This interesting substance has also been seen in much larger amounts in a pathological urine (liver disease) and would seem to repay further investigation. Another substance giving a bright red colour like histidine but with an R_F value of 0·30 has also been found in similar urines. Rough matching showed that this may be histamine. The collidine strip method is the most simple and specific general method known to the writer for the micro detection of histidine and tyrosine in the presence of each other.

The presence of methionine sulphoxide in the urine of E.C. was shown only after some difficulty and it is still not possible to say whether any of it is present as such in the fresh urine, or whether it has all been formed as an artifact by oxidation of methionine during storage of the urine or preparation of the chromatogram. The histidine spot often appeared to be double, the slower moving part in the phenol being greenish (like the colour given by histidine) and the faster part being purple. Careful matching experiments have shown that the faster part of the spot was due to methionine sulphoxide, which is not therefore clearly distinguishable on the chromatogram from histidine. The diazo reaction is also of value in this differentiation as, of course, only histidine gives a positive result. On the samples of urine from E.C. after methionine feeding it was thus possible to show that the large increase in size in the histidine + methionine sulphoxide spot was due entirely to the increased excretion of the latter compound, the histidine excretion remaining constant. At other times however the spot was chiefly contributed by histidine.

It was not possible to identify the substance called 'over-glycine' (Fig. 1). It was always very weak and so its appearance or otherwise in a given urine was within the

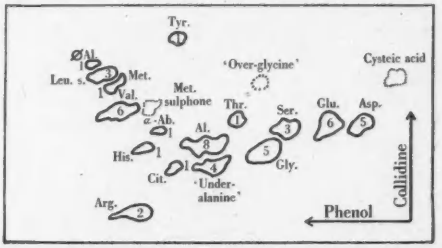


Fig. 1. Tracing showing the positions of the amino-acids in a typical urine of E.C. The figures inset represent relative colour strengths on an arbitrary scale. 'Over-glycine' is usually very weak. Methionine sulphone and cysteic acid are regularly seen if the urine is previously treated with H_2O_2, but the former is very weak except after methionine feeding. Methionine sulphoxide occurs just to the left of histidine. øAl. = phenylalanine. α-Ab. = α-amino-butyric acid.

range of experimental error. A very similar if not identical substance occurs in greater amounts in other pathological urines, and has been shown to be stable to hydrolysis. It is therefore believed to be due to an unknown amino-acid.

The rough quantitative estimation of amino-acids was done by matching the size and strength of the ninhydrin colours by trial and error against synthetic mixtures. The urine of E.C. passed on 7 Jan. 1946 was taken for this purpose. It is not claimed that the accuracy of this method is greater than $\pm 30\%$. Only weak colours should be compared, the urine being diluted if necessary. Marked daily variations of the outputs of some amino-acids occurred.

For chromatography the serum or plasma (Fig. 2) was treated with 10 times its volume of 95% ethanol, filtered and finally concentrated to one-tenth of the original volume before taking 25μl. as usual for the chromatogram. This method was chosen in order to avoid more reactive protein precipitants which might hydrolyze peptides.

Many other substances previously reported to react with ninhydrin (e.g. ammonium salts, reducing agents) have

been run on the chromatograms but (except for β-alanine and glucosamine, see Table 6) no reaction resembling that obtained with α-amino-acids and peptides has been encountered.

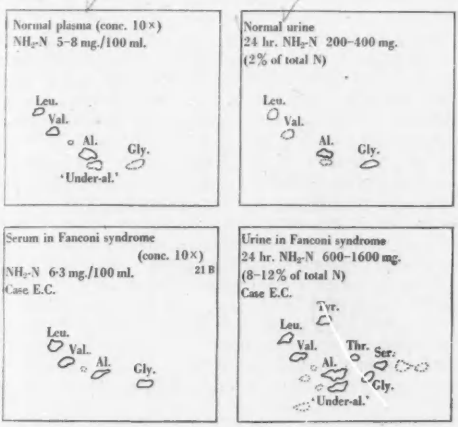


Fig. 2. Four tracings showing main amino-acids in plasma (or serum) and in the urine of normals and in Fanconi syndrome. The outline of the weaker spots is dotted. α-Amino-*n*-butyric acid is seen between alanine and valine.

It may be added here that the use of small squares of filter paper (12 × 12 cm.) shows promise as a rough method. Only 3 μl. of urine is required and they take only 2–3 hr. to run with each solvent.

Other urinary constituents on the chromatograms

To determine the movement of these on the chromatograms synthetic mixtures were made up of all the main constituents of normal urine, in the strength in which they usually occur (see Fig. 3) as well as of glucose in the concentration occurring in the urine of E.C. Each solution (25 μl) was then run for a distance of 25 cm. on the one dimensional chromatogram with phenol and again on another strip with collidine. The position, if any, taken up by the substance in question was determined by the use of specific developers. Sodium hippurate however could not be developed at the normally occurring strength so the concentration had to be increased to 1% (w/v). The developers are tabulated in the previous column. The results are shown on Fig. 3. In addition a separate set of phenol chromatograms of all the above constituents was run and the strips sprayed with ninhydrin and heated at 100°. No coloured bands of any kind appeared.

Substance	Method of development	Result
All chlorides	Dip into 0·1 N-$AgNO_3$, wash with distilled water, expose to light	Black band
Sulphate and phosphate	Dip into saturated basic lead acetate solution, wash, expose to H_2S	Brown band
Uric acid	Dip quickly into 1% ammoniacal $AgNO_3$	Black band
Glucose	Dip quickly in 1% ammoniacal $AgNO_3$, hang up in dark to dry, then heat to 100° for 10 min.	Black band
Creatinine	Picric acid, NaOH (Jaffé reagent)	Red band
Urea	Apply streaks of 10% $Hg(NO_3)_2$ to the paper, followed by streaks of 2 N-NaOH	Colourless band on grey background
Na hippurate	Observe in ultra-violet light	Yellowish white fluorescence

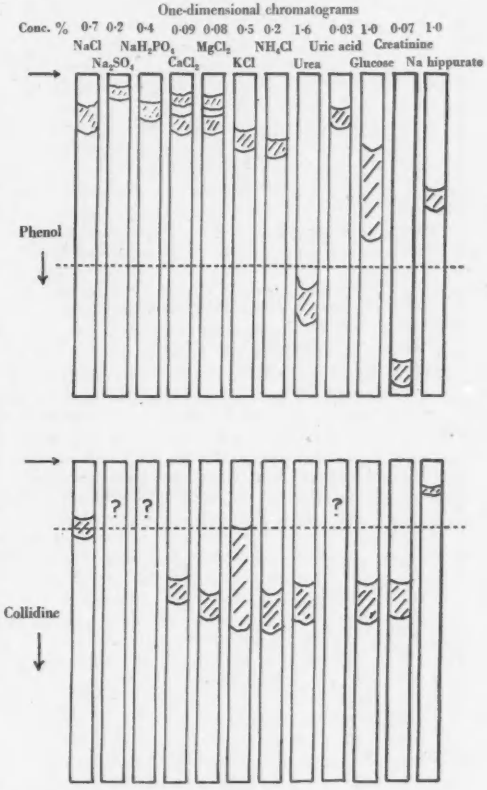
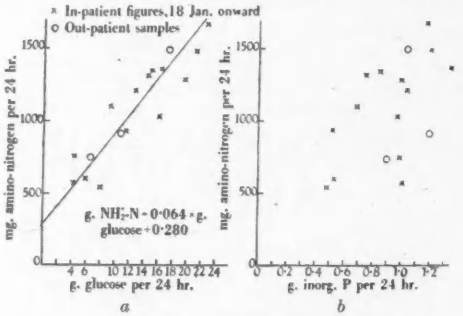


Fig. 3. These drawings are to scale with respect to the position and size of the bands. The substance in question has been placed at the top end of the strip and the solvent has moved to the extreme end of it. The three strips marked '?' gave no bands in collidine. The salts with divalent kations (Ca, Mg) gave double bands in phenol. The slower band in each case was the stronger and sharper. The theoretical position of 'under-alanine' in both chromatograms is marked with a dotted line.

It is interesting to remark here that many other substances in pathological urines have also been shown to move characteristically on the chromatograms, e.g. homogentisic acid, melanogen, a substance giving a red ultra-violet fluorescence resembling that of a porphyrin, and lactose.


× In-patient figures, 18 Jan. onward
○ Out-patient samples
mg. amino-nitrogen per 24 hr.
1500
1000
500
0
g. NH_2-N = 0·064 × g. glucose + 0·280
4 6 10 12 14 16 18 20 22 24
g. glucose per 24 hr.
a
0 0·2 0·4 0·6 0·8 1·0 1·2
g. inorg. P per 24 hr.
b

Figs. 4*a* and *b*. Case E.C. Urine analyses.

Hydrolysis of urine

The urine was mixed with an equal volume of conc. HCl and refluxed for 24 hr. It was then evaporated to dryness *in vacuo* and again evaporated 3 times after addition of 10 ml. of water. The residue was made up to the original urine volume and 25 μl. taken for the chromatograms as usual. The coloured impurities present did not interfere in any way. More than the merest trace of protein was never present. No attempt was made to remove this first.

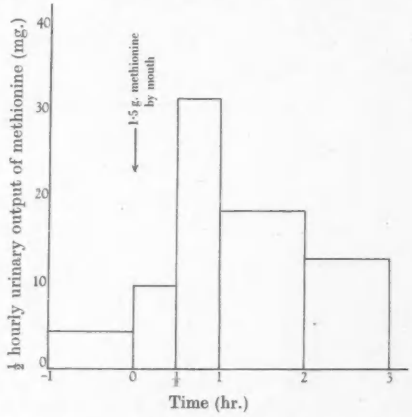


Fig. 5. Normal methionine saturation curve given by E.C.

Isolation and reactions of 'under-alanine'

Attempts to match this spot (see Fig. 1) with any known amino-acid or other substance which might react with ninhydrin to give a colour all failed. The substance gave a ninhydrin reaction slowly at room temperature. The colour closely matched the slightly reddish purple given by glycine. The possibility that the spot might be due to an artifact was considered since it usually has very much the same size and shape as the alanine spot just above it. However it always appeared when the chromatogram was repeated and whatever the order of the solvents used. Running the chromatogram in the presence of vapour of HCl or NH_3 had but little effect on the R_F values so the substance was not likely to be basic or acidic. Hydrolysis of the urine, however, caused its complete disappearance while all the other spots remained (Table 4). It must therefore be a peptide. From Fig. 1 it can be seen that its isolation would not be possible by a one-dimensional method using either of those solvents. From Fig. 3 it can be seen that none of the common urinary constituents move at the same speed in both solvents. They would not therefore contaminate the 'under-alanine' spot on the paper. It was decided to attempt the separation of this component by a two-dimensional method rather than experiment with unfamiliar solvents which would entail re-determination of all the R_F values in question. The following method was used:

On two large squares (22 × 18 in.) of No. 1 Whatman filter paper along a line 6 cm. from one long edge 28 spots, each from 25 μl. of the urine of E.C. passed on 7 Jan. 1946, were placed at regular intervals (see Fig. 6, A). The sheets

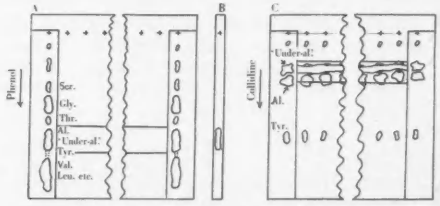


Fig. 6. The crosses in A represent places where 25 μl. of urine were placed, in B and C where the solution containing alanine, 'under-alanine' and tyrosine was placed after washing from the appropriate piece of A. The spots outlined represent amino-acids developed with ninhydrin to serve as markers. A and B were run with phenol, B serving to confirm lack of contamination with adjacent amino-acids. In C which was run with collidine the final isolation of pure 'under-alanine' was performed (see text).

were run with phenol in the chromatogram cabinet in the usual way. On one sheet three, and on the other four, strips of paper each corresponding to the run of a drop of urine were cut out, developed with ninhydrin and then reinserted into the sheets in their original positions, so as to serve as markers. The pieces of paper on the rest of the sheet corresponding to the developed positions of alanine, 'under-alanine' and tyrosine on the markers, could readily be marked out and cut off. The papers were washed by soaking water along them in the apparatus shown in Fig. 7 which was very convenient for the purpose. After some hours the washing was stopped and the paper dried and developed with ninhydrin to confirm removal of all the amino-acids. This always occurred readily and it is believed that these amino-acids moved forward almost as fast as the edge of advancing water. The combined wash waters were evaporated to dryness and made up to 525 μl. with distilled

water, i.e. to the volume of 21 spots of 25μl. of original urine. Of this 25μl. was run on a phenol strip (Fig. 6, B). It gave when developed with ninhydrin a single spot corresponding in position and intensity to the amino-acids cut out.

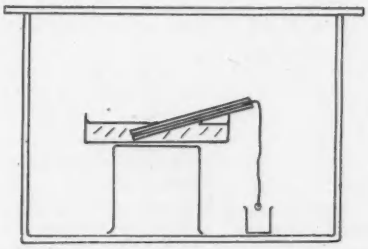


Fig. 7. The piece of filter paper containing the required amino-acids is pinched as shown between two pieces of glass resting in a Petri dish. The water in the dish rises by capillary attraction between the plates and is thus supplied to the filter paper. The wash liquor eventually drops off the end of the paper into the little beaker. Evaporation must be prevented by the larger outer vessel.

Two further sheets of paper (Fig. 6, C) were taken for the collidine run. The remaining 20 lots each of 25μl. of solution were placed parallel to the shorter edge of the paper so as to have a maximum length of run. After running with collidine to the full length of the paper it was removed and dried. The two lateral strips of each sheet were cut away, developed with ninhydrin and reinserted. This time the 'under-alanine' could not be cut away as before owing to the very small space, about 4 mm., between it and the alanine spot, this space often being curved. It was isolated as shown by cautiously cutting away strips of paper and developing with ninhydrin until the entire alanine spot and the two extreme edges of 'under-alanine' could be accounted for. This was the only stage at which any definite loss of material is believed to have taken place. The pure 'under-alanine' was washed off the paper as before. Evaporation of the solution left a minute residue of small stumpy needles not quickly soluble when they were made up to a volume of 400μl. (i.e. $16 \times 25\mu$l.) with water. This was the stock solution for the experiments described in the following paragraphs.

Check for purity. 25μl. of solution were run on the two-dimensional chromatogram. A spot corresponding exactly to 'under-alanine', but weaker than from 25μl. of original urine, was obtained together with the merest visible trace of glycine. Glycine is not likely to have been present from an error in cutting the chromatogram as it is some inches away. It is more likely to have appeared from decomposition of 'under-alanine'. The absence of any alanine as impurity was particularly gratifying.

Hydrolysis. 25μl. of solution were sealed in a capillary glass tube with 25μl. of conc. HCl. After 24 hr. in boiling water the liquid was transferred to a watch glass and evaporated *in vacuo*. This was repeated after addition to the residue of a drop of water. The crystalline residue was readily soluble in water. It was transferred to the appropriate position on a large square of filter paper and a two-dimensional chromatogram made. After development it showed two strong spots in the positions of serine and of glycine. The strengths of the spots corresponded to about 4–6μg. of serine and 5–9μg. of glycine. From these quantities found in 25μl. of original urine, the concentration of 'under-alanine' in the urine must be at least 400 mg./l. The molecular ratios are very close to 1 serine : 2 glycine.

Final identification of hydrolysis products. The above experiment was repeated with 4μg. of serine and 3μg. of glycine added to the mixture on the chromatogram. After development the sheet showed only serine and glycine, the markers having coincided exactly with the hydrolysis products.

End-group determination. This was done in the following three ways:

(*a*) 25μl. of solution were mixed with 5μl. of 0·1 N-$NaNO_2$ and 25μl. of conc. HCl, the latter being added very slowly. After 15 min. at room temperature, a small crystal of urea was added to destroy the remaining HNO_2 and the whole transferred to a sealed tube and heated at 100° for 16 hr. After removal of the acid as above it was run on the chromatogram. It showed one strong spot corresponding to about 5–9μg. of glycine.

(*b*) 25μl. of solution were mixed with 5μl. of 1% $NaHCO_3$ and 5μl. of 4% fluorodinitrobenzene in ethanol (Sanger, 1945) and sealed in a capillary tube. Most of the reagent precipitated at once as oily drops. The capillary tube was mounted obliquely in a test tube and the whole turned 3 hr. on a roller mill, this giving good stirring up of the oily drops. The capillary was then opened, the contents transferred to a watch glass and evaporated to dryness *in vacuo*. The residue was washed with ether till no oil remained, and the yellow crystals remaining were taken up in 50μl. of 6 N-HCl, sealed in a capillary tube and heated for 2 hr. at 100° (dinitrophenylglycine, if present, is hydrolyzed appreciably on longer heating under these conditions). After removal of the acid as usual, it was run on the chromatogram. A fast moving yellow spot presumably of the dinitrophenyl derivative of the end amino-acid was seen during the phenol run. About 4–7μg. of glycine only were found on the final chromatogram.

(*c*) 25μl. of solution were mixed with 5μl. of 10% $NaHCO_3$ and 20μl. of 1% KIO_4. After 30 min. at room temperature 20μl. of 1% tartaric acid were added to decompose the excess KIO_4. After another 10 min., 10μl. of 8·25% KI and 40μl. of 2 N-HCl were added. The copious precipitate of I_2 was taken up by cautious addition of an excess of Na_2SO_3 solution (it would have been better to allow most of the I_2 to evaporate first). An equal volume of conc. HCl was added and the whole heated at 100° in a capillary tube for 16 hr. After removal of the acid as usual the residue was transferred to the chromatogram without any attempt to remove the remaining inorganic substances. About 5–9μg. of glycine only was found.

Check on the technique. Pure *l*-seryl-glycine (from Dr J. S. Fruton) had R_F values in phenol and collidine quite different from those of 'under-alanine' (Table 6). It was hydrolyzed as above and run on the chromatogram. The spots were roughly matched and found to contain about 3μg. of serine and 2·2μg. of glycine which gives a molar ratio of 1 : 1. This was unexpectedly close as the accuracy of the matching is not good. However, this experiment shows that the method is of some value and that the relative proportions of serine and glycine are not appreciably

changed by decomposition under the conditions of the hydrolysis. It must be added, however, that the overall yield of serine and glycine from the pure seryl-glycine used was only about 50%.

Reactions of reducing substance in the urine. The urine of 7 Jan. 1946 (case E.C.) was taken for these tests. It gave a red precipitate on boiling in the usual manner with Benedict's qualitative reagent. It also gave a typical glucosazone (m.p. 207°) and a positive fermentation test for glucose performed as described by Harrison (1937). Selivanoff's test was negative.

RESULTS

These are given in the appropriate Tables and Figs.

Other analyses (Case E.C.)

These will be described in detail for the blood and urine by Stowers & Dent (1947). A brief summary of typical findings in the case E.C. is given for completeness. Urobilin and bilirubin were tested for repeatedly in the urine throughout the illness. Urobilin was present in slight excess throughout, bilirubin was never found (Fouchet test), this in spite of the slightly yellow appearance of the sclera of the patient in the last few days of life.

Date	
29. iv. 45	Glucose tolerance curve normal with sugar in all specimens of urine
7. viii. 45	Alkaline phosphatase 63 units (King & Armstrong). Alkali reserve 56 vol. %
16. vii. 45	Ammonia coefficient of urine 32%
18. ix. 45	Plasma inorganic phosphorus 1·8 mg./100 ml.
18. ix. 45	Serum calcium 11·3 mg./100 ml.
19. i. 46	Blood urea 39 mg./100 ml.
23. i. 46	Serum amino nitrogen 6·3 mg./100 ml.

X-rays of the skeleton showed marked generalized osteomalacia, with numerous pseudo-fractures without callus formation, tri-radiate pelvis, 'fish disc' lumbar vertebrae.

The results of the rough quantitative estimation of separate amino-acids are given only in the summary. They refer to the 24 hr. urine of 7 Jan. 1946.

The amino-aciduria was first tested for and found in July 1945. It occurred in every sample of urine tested subsequently until the death of the patient in Feb. 1946.

DISCUSSION

The individual points set out for study are reviewed under the following headings.

The nature and extent of the amino-aciduria. The normal variation in daily output of amino nitrogen in the urine is considerable. The question is well reviewed by Kirk (1936) who also adds the results of many further investigations of his own upon both normal subjects and patients with liver disease. The best method of determination is the modified formol titration (Van Slyke & Kirk, 1933) and 24 hr. outputs determined this way usually vary from 100 to 400 mg. which is 1–2% of the total nitrogen. There is no significant difference in cirrhosis of the liver, infective hepatitis and obstructive jaundice.

Albanese & Irby (1944), using a different and simpler technique depending on the volumetric determination of copper in the soluble amino-acid copper salts, find consistently higher results. In 15 normal men the amino nitrogen output was 200–700 mg./24 hr. which was 3–4% of the total nitrogen. This method was avoided here as it has not yet been compared adequately with the formol method and moreover it has limitations due to the comparative insolubility of certain amino-acid copper salts.

The daily excretion of an average of *c.* 1050 mg. of amino nitrogen in the case E.C. (see Table 1) can only be considered as grossly abnormal. The daily variation was considerable, from 491 to 1655 mg., the concentration varied from 44 to 116 mg./100 ml. and the ratio NH_2-N/total N from 3·1 to 13·0. The latter figures can be compared with those of 4–16 found by Stadie & Van Slyke (1920) in a case of acute yellow atrophy. Table 1 shows that this ratio is the most constant and therefore probably the most significant of the various methods of expressing the amino nitrogen output, especially if the low figures for the Oct. and Nov. samples are set aside. It is suggested that it is this figure which should always be determined when any case of excessive amino-aciduria is under consideration. Probably it is safe to say that any value greater than 3 is abnormal when determined by the formol titration. It is noticed in the case E.C. that although there was probably a tendency for the amino-acid output to rise from Oct. to Jan. yet there was no sudden rise in the last days before death nor indeed in the last month of life. Whatever the significance of the amino-aciduria it was not closely linked to the process causing death. The contrast with the terminal rise in urinary amino-acid output in the last few days of fatal acute yellow atrophy of the liver could not be more marked.

Unfortunately it was possible to determine the protein intake of E.C. over a period of only 7 days. There was then but little correspondence with amino nitrogen output in the urine (Table 1). During the last days of life, hardly any food was taken but the total nitrogen and amino nitrogen outputs both rose.

The large day to day variations in total amino nitrogen in the urine still remain very puzzling. Promoting a diuresis by drinking water did not significantly alter the total amount excreted (22nd Jan.). When however a spontaneous diuresis occurred so that fluid output was greater than fluid intake, the total excretion of amino nitrogen rose

Table 1. *Case E.C.* 24 *hr. output of urinary constituents*

Date	Urine (ml.)	Total N (g.)	Total NH_2-N (mg.)	NH_2-N (mg./100 ml.)	(NH_2-N/total N) ×100	Total S (mg.)	Total N/total S	Glucose (g.)	Inorg. P (g.)	Total N/total P	Protein eaten (g.)	Remarks
16 Oct.	1585	12·40	740	46	3·8	717	17·3	6·4	0·90	13·8	—	
18 Nov.	2080	14·35	910	44	3·1	—	—	10·7	1·20	12·0	—	
7 Jan.	1320	13·75	1480	112	8·1	—	—	17·2	1·03	13·3	—	
18 Jan.	940	8·66	1090	116	12·6	610	14·2	9·1	0·69	12·6	—	
19 Jan.	2000	12·15	1524	76	12·5	749	16·2	—	—	—	—	3 pints of blood transfused
20 Jan.	2220	9·88	1123	51	11·4	543	18·2	—	—	—	—	
21 Jan.	1935	10·81	1365	71	12·6	676	16·0	—	—	—	—	
22 Jan.	1035	5·50	491	47	8·9	334	16·4	—	—	—	—	Water excretion test, 1 l. of water drunk
23 Jan.	1910	10·72*	749*	39	7·0	782	13·4	4·2	0·99	10·8	75	
24 Jan.	1950	13·18*	1646*	84	12·5	1665	8·0	—	—	—	84	10 g. of *dl*-methionine by mouth
25 Jan.	1060	12·91	560	53	4·3	1086	11·9	4·1	1·01	12·8	30	(Urines preserved with HCl from now on). Haematemesis, 400 ml.
26 Jan.	1370	8·67	826	601	9·5	591	14·7	—	—	—	53	
27 Jan.	1610	10·30	947	59	9·2	731	14·1	—	—	—	74	
28 Jan.	828	5·72	499	60	8·7	380	15·0	—	—	—	60	5 g. of *dl*-serine by mouth
29 Jan.	815	5·25	592	73	11·3	319	16·4	5·8	0·51	10·2	54	
30 Jan.	850	4·89	531	62	10·9	296	16·5	7·9	0·49	10·0	—	
31 Jan.	1250	7·53*	892*	71	11·9	740	10·2	—	—	—	—	8·05 g. of *l*-cystine by mouth
1 Feb.	1400	11·12	1305	93	11·7	1980	5·6	14·4	0·75	14·8	—	Haematemesis
2 Feb.	1670	14·03	1200	72	8·5	1160	12·1	12·3	1·04	13·5	—	
3 Feb.	2150	15·10	1655	77	11·0	1000	15·1	22·6	1·17	12·9	—	
4 Feb.	1950	19·01	1350	69	7·1	1280	14·8	16·2	1·34	14·2	—	
6 Feb.	1600	15·70	1275	80	8·1	1055	14·9	19·5	0·99	15·9	—	Noon to noon urine collection started
7 Feb.	1530	14·59	1021	67	6·8	1050	13·9	16·0	0·97	15·0	—	
8 Feb.	1315	10·24	1335	104	13·0	1710	6·0	14·9	0·85	12·1	—	10 g. of *dl*-methionine by mouth
9 Feb.	2300	14·79	1475	63	10·0	1820	8·1	21·0	1·20	12·3	—	Haematemesis, 200 ml. 2 pints of blood transfused
10 Feb.	1285	8·45	929	72	11·0	778	10·9	11·4	0·52	16·2	—	In coma
11 Feb.					Incontinent, no samples							
12 Feb.	(100)	(1·19%)	—	103	8·7	(0·054%)	21·8	—	—	—	—	Catheter specimen of urine
13 Feb.	(90)	(1·14%)	—	83	7·3	(0·055%)	20·7	—	—	—	—	Died 4 a.m. Urine from bladder at post-mortem

* Duplicate determinations.

Table 2. *Case E.C. Output of urinary constituents after amino-acids by mouth*

Date	Time (hr.)	Urine (ml.)	Total N (g.)	Total NH_2-N (mg.)	NH_2-N (mg./100 ml.)	(NH_2-N/total N) ×100	Total S (mg.)	Total N/total S	SO_4-S (mg.)	(SO_4-S/total S) ×100	Inorg. P (g.)	Total N/total P	Amino-acid given
24 Jan.	0– 3	190	1·31	291	153	22·1	229	5·7	83	36·4	—	—	10 g. *dl*-methionine
	3– 6	210	1·42	222	106	15·6	226	6·3	128	56·6	—	—	
	6–24	1550	10·45	1133	73	10·8	1210	8·6	880	72·7	—	—	
25 Jan.	0–24	1060	12·91	560	53	4·3	1086	11·9	843	77·6	—	—	
28 Jan.	0– 3	128	0·99	185	144	18·7	61	16·2	—	—	0·074	13·4	5 g. *dl*-serine
	0– 6	125	0·94	124	99	13·3	56	16·8	—	—	0·057	16·5	
	6–24	575	3·79	190	32	5·0	263	14·4	—	—	0·340	11·1	
29 Jan.	0–24	815	5·25	592	73	11·3	319	16·4	—	—	0·515	10·2	
31 Jan.	0– 3	290	1·73	177	61	10·4	110	15·6	80	72·0	—	—	8·05 g. *l*-cystine
	3– 6	200	1·28	154	77	12·0	128	10·0	97	76·1	—	—	
	6–18	760	4·52	561	74	12·4	501	9·0	382	76·3	—	—	
1 Feb.	0–24	1400	1·11	1305	93	11·7	1980	5·6	1640	82·8	—	—	
2 Feb.	0–24	1670	1·40	1200	72	8·5	1160	12·1	813	70·1	—	—	

N.B. Only 24 hr. urines were collected after the second 10 g. of methionine on 8 Feb. 1946.

(Fig. 8). Such diureses frequently occurred in this patient whose ascites and oedema varied from time to time. The tentative suggestion follows that the spontaneous diureses represent excretions of excesses of tissue fluids in which the amino nitrogen and total non-protein nitrogen exist in the proportions found in the urine.

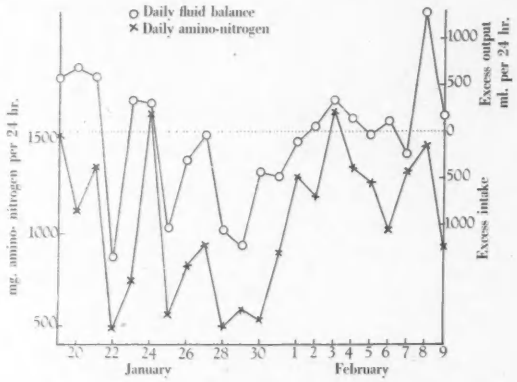


Fig. 8. Curve to show relationship between fluid balance and 24 hr. output of amino nitrogen (case E.C.). Fluid balance was measured by the nursing staff as the difference in volume between total fluid intake and urine output.

The other cases of Fanconi syndrome have shown lower outputs of amino nitrogen (Table 3). However, the output is considered abnormal in both, largely on the basis of the raised NH_2-N/total N ratios of 3·6 and 4·8 in V.R. and E.B. respectively.

Table 3. *Urine in other cases of Fanconi syndrome*

Date	N (g./100 ml.)	NH_2-N (mg./100 ml.)	(NH_2-N/total N) × 100
	Case V.R.		
2 Oct. 1945	1·09	39	3·6
	Case E.B.		
13 Mar. 1946	0·92	44	4·8

In the former, terminal renal failure with nitrogen retention may have affected the output. (Compare the case of acute yellow atrophy with grossly raised blood amino nitrogen and urinary retention; Rabinowitch, 1928.) In E.B. who is still fairly healthy, the amino-aciduria was intermittent and was only found in one of 4 samples submitted. It does appear from this and from the lower ratios of the Oct. and Nov. samples of the case E.C. that, taken over a period of some months, there is a suggestion that the amino-aciduria increases as the disease progresses. It must be pointed out that all three cases had cirrhosis of the liver, E.C. and V.R. also with primary carcinoma. Cirrhosis in itself, however, does not produce an amino-aciduria (Kirk, 1936) and this has recently been confirmed by the author, using chromatograms, for a small series of cirrhoses and secondary carcinomata of the liver.

With regard to the individual amino-acids found in the urine, it is seen that all those commonly found in protein hydrolysates except lysine and tryptophan have been detected in the urine of E.C. at some time or another, and for technical reasons it is not possible to say whether both leucine and isoleucine, or only one of these, are present. Proline has been seen only once, in the urine of 9 Feb. 1946. In addition citrulline and α-amino-*n*-butyric acid have been identified as occurring in small but fairly constant amounts. Methionine sulphoxide and cysteic acid were found occasionally, but could have been produced by oxidation under the conditions of storage. Methionine sulphoxide is a particularly interesting substance from its potential importance in connexion with oxidation-reduction potential. It oxidizes cysteine to cystine (Toennies & Kolb, 1939). It will be important if it can be shown that it occurs naturally for it could then act as an oxygen carrier. One strong spot on the chromatogram, 'under-alanine', is believed to be due to a considerable excretion of a peptide which liberates serine and glycine on hydrolysis and is probably seryl-glycyl-glycine, though this requires confirmation by synthesis. Another weak but fairly constant spot, 'over-glycine', is believed to be due to a new amino-acid. From its position on the chromatogram it can be surmised that it is probably of novel composition. A higher homologue of glutamic acid would be expected to occupy such a position. It is a pity that the occurrence or otherwise of asparagine and glutamine cannot be definitely inferred by partition chromatography in the presence of glycine and alanine, with both of which they overlap. This is likely to be a permanent defect in the method as it is to be expected that the partition coefficients in any other solvents would be similar, the contribution to them of the —CO.NH_2 group being similar to that of the hydrogen atom (McMeekin, Cohn & Weare, 1936). It can be said that there are unknown substances present which set free large amounts of aspartic and glutamic acids on hydrolysis. No otherwise suggestive evidence of the presence of asparagine and glutamine has been obtained.

The naming of new spots by the prefixes 'under' and 'over' with respect to the position of known amino-acids has been found very convenient as the regular positions of the common amino-acids soon become very familiar to anyone working with the chromatograms. Spots to the right or left are named with the prefixes 'slow' or 'fast' respectively.

this referring to their speeds in the phenol solvent; e.g. 'fast-glycine' is a new spot found in hydrolyzed urines which is still undergoing investigation. It occurs just to the left of the glycine spot on the chromatograms as drawn in this paper.

A glance at the chromatogram of the urine gives at once an accurate qualitative and rough quantitative estimate of amino-acid outputs (Table 4). However, as the standard amount of 25 μl. of urine is taken each time, it is the concentration and not the total 24 hr. output of amino-acids that is being estimated. The amino-acid pattern was remarkably constant over long periods. Only aspartic and glutamic acids varied considerably from day to day (see Table 4, case E.C.), if the days following ingestion of methionine and serine are excluded. When a lower concentration of amino nitrogen was passed by E.C. the reduction chiefly affected the amino-acids of higher molecular weight (valine and leucine especially), in fact the chromatogram began to resemble that of V.R. and E.B. At first 'under-alanine' was found to be a constant constituent but later on it was occasionally absent. It is believed more likely that it was originally present in all the urines of E.C. and that during the wait of some months in an imperfect refrigerator partial hydrolysis to glycine and seryl-glycine occurred. The latter would not show as a separate spot on the chromatogram owing to its relatively poor strength of colour with ninhydrin, and is itself stable enough to resist further mild hydrolysis. The additional glycine would not be very conspicuous on an already strong spot. It can be noted that in those urines in which no 'under-alanine' was found the aspartic and glutamic acid contents were invariably high, these also being liberated by hydrolysis. The two chromatograms made on the urine of 13 Feb. also confirm this (Table 4). It will be necessary in future to guard against such instability by greater precautions in storage. The urine of E.B. gave a chromatogram not far removed from those characteristic for the weaker ones of E.C. That of V.R. was similar except that 'under-alanine' was absent and serine much stronger than usual. Here it is believed that complete hydrolysis of the 'under-alanine' to serine and glycine had taken place under the conditions of preservation in dilute hydrochloric acid at room temperature.

Nearly all the time E.C. was an in-patient he was on a diet of milk as sole source of protein. Casein, hydrolyzed with acid and run on the chromatogram in comparable amount, gave a distinct picture quite unlike that given by the urine before or after hydrolysis. E.C. was on a mixed diet as an out-patient but the urinary amino-acid distribution was unchanged. There therefore is no suggestion of the urinary amino-acids representing a simple overflow of the amino-acids from ingested protein, such as might have escaped the attention of the liver owing to the portal obstruction and consequent anastomoses with the systemic circulation. The excretion was selective.

The question of the abnormal distribution of the amino-acids in the urine of E.C. has now to be considered. Many normal urines have been investigated by chromatography and at the normal concentrations of 10–30 mg. of amino nitrogen/100 ml. the main amino-acids seen on the chromatogram are glycine and alanine. Traces of glutamic acid, valine and 'under-alanine' sometimes appear. Concentration of the urine leads to difficulties owing to the large amounts of other substances present which then begin to interfere with the development of the chromatogram, and the methods of removing these so far tried (ion exchange, solvent extraction, etc.) tend to alter the relative amounts of the amino-acids present. Leucine, histidine, tyrosine, citrulline, serine, 'under-alanine' and α-amino-*n*-butyric acid have however been demonstrated at one stage or another in normal urine, but it is impossible to say definitely whether the relative amounts of the various amino-acids present are similar to those found in Fanconi syndrome. The general impression is that in the latter there is a relative increase in the amounts of dicarboxylic acids liberated on hydrolysis, and of hydroxyamino and higher molecular weight monoaminomonocarboxylic acids. This is well shown if a normal urine is compared against a urine from Fanconi syndrome which has been diluted to a comparable concentration of amino nitrogen, but this procedure does not allow the strengths of the weaker spots to be compared.

The mechanism of the amino-aciduria. Some factors already considered above have suggested that a simple overflow of amino-acids from the blood into the urine as in acute yellow atrophy is not a likely mechanism. This is finally confirmed by the finding of a normal value for serum amino nitrogen (6·3 mg./100 ml.). There must therefore be some form of lowered renal threshold, presumably a defective reabsorption of amino-acids in the tubules from the glomerular filtrate. Fig. 2 attempts to show diagrammatically that although the serum amino-acid distribution in the case E.C. was very similar to the normal serum and to a normal urine (which therefore resembles a simple plasma filtrate), yet the urinary distribution of E.C. was quite distinct, thus suggesting that the tubular defect is of different degree for different amino-acids. It is interesting to note that both 'under-alanine' and α-amino-*n*-butyric acids have been definitely identified in normal blood and urine as well as in the Fanconi syndrome.

The relation between the amino-aciduria and the glycosuria. Fanconi (1936), who found that the glycosuria in his cases was renal in type, was the first

Table 4. *Case E.C. Two-dimensional chromatograms of the urine*

(Day to day strengths of amino-acid ninhydrin reactions on an arbitrary scale of 10 units. 1 unit = faint colour, 10 = intense colour. 25μl. of urine taken in each case.

Date	Aspartic acid	Glutamic acid	Serine	Glycine	Threonine	Alanine	'Under-alanine'	Citrulline	Histidine + methionine sulphoxide	α-Aminobutyric acid	Hydroxyproline	Valine	Leucine and/or isoleucine	Methionine	Phenylalanine	Tyrosine	'Over glycine'	Arginine	Remarks
16 Oct.	1	1	5	7	1	7	7	1	1	—	—	2	1	—	—	<1	1	<1	
18 Nov.	—	—	2	6	2	6	5	—	2	1	—	2	2	—	—	<1	<1	—	
7 Jan.	—	1	4	6	2	8	7	1	2	2	<1	5	4	—	<1	2	—	1	
	7	8	>10	8	3	10	—	<1	<1	1	2	5	4	—	1	2	—	<1	Urine hydrolyzed 2 hr. at 100° with 6N-HCl
18 Jan.	4	4	2	5	1	8	—	1	<1	2	—	3	3	—	—	2	1	1	
23 Jan.	2	6	2	5	<1	10	—	<1	<1	<1	—	1	1	—	—	<1	—	—	
24 Jan.	3	6	3	6	1	8	—	1	6	3	2	4	3	5	—	<1	—	—	0–3 hr. after methionine
	2	7	4	6	1	7	—	2	6	3	—	6	3	8	1	2	<1	1	3–6 hr. after methionine
	6	7	3	5	1	10	—	1	4	2	—	5	1	2	—	1	<1	1	6–18 hr. after methionine
25 Jan.	—	3	2	6	<1	6	1	—	1	1	—	1	1	—	—	<1	<1	—	
28 Jan.	—	—	⩾10	7	2	7	6	1	1	2	—	3	2	—	—	1	—	—	0–3 hr. after serine
	3	7	7	7	1	7	—	1	1	1	—	4	2	—	1	2	<1	1	3–6 hr. after serine
29 Jan.	—	3	5	6	1	7	3	2	<1	1	—	4	2	—	1	1	1	1	
31 Jan.	1	4	3	6	1	6	2	1	2	<1	—	3	2	—	—	<1	<1	1	0–3 hr. after cystine
	—	1	3	5	1	6	4	1	1	1	—	3	1	—	—	2	—	<1	6–18 hr. after cystine
1 Feb.	3	—	2	5	1	7	—	1	1	1	—	2	2	—	—	1	—	—	
2 Feb.	3	4	3	4	1	7	—	1	1	1	—	3	2	—	—	1	<1	<1	
3 Feb.	5	7	4	6	2	6	3	2	1	2	—	4	3	—	—	—	1	<1	
7 Feb.	—	—	2	5	1	5	4	1	1	1	—	3	1	—	—	1	—	—	
8 Feb.	—	—	3	6	<1	7	4	1	4	2	—	3	1	2	—	1	1	—	0–24 hr. after methionine
9 Feb.	—	<1	4	7	2	8	4	2	3	1	—	5	2	—	—	1	3	1	
12 Feb.	—	—	4	5	2	7	6	1	1	2	—	6	4	—	2	3	<1	1	Catheter specimen
13 Feb.	—	—	3	6	1	7	5				Spoilt					2	—	—	Post-mortem specimen. Fresh
	5*	7	3	6	1	6	—	?*	1	2	—	4	2	—	1	3	<1	1	Post-mortem specimen. After 4 months in refrigerator

* N.B. The second specimen for 13 Feb. was developed some months after the first. Pure citrulline was added to it to serve as a marker so the strength of the original spot cannot be ascertained.

Case V.R. 20 Oct.	<1	2	6	5	1	4	—	—	2	—	—	—	—	—	—	—	—	—	Urine taken during last 2 days of life. Some renal failure developing
Case E.B. 13 Mar.	—	—	2	5	<1	3	2	—	4	<1	—	—	—	—	—	—	<1	—	

N.B. The strength of the ninhydrin reaction does not give a direct measure of amino nitrogen, unless only the same amino-acid is being considered. The above figures can be compared vertically but not horizontally.

to suggest a similar mechanism for the amino-aciduria. The case E.C. also showed a renal glycosuric blood-sugar curve and it was very early noticed on qualitative testing of the Oct., Nov. and early Jan. urine specimens that the glycosuria increased with the increasing amino-aciduria. Quantitative determinations of glucose output were later done on all the available specimens of urine and the results plotted against the corresponding amino nitrogen output are shown in Fig. 4A. There appears to be a simple straight line relationship between the two which can be expressed by the formula:

$$\text{g. } NH_2\text{-N} = 0{\cdot}064 \times \text{g. glucose} + 0{\cdot}280.$$

The formula agrees well over a fivefold range of glucose outputs. The inference cannot be avoided that there is some close connexion, previously unsuspected, between the actual chemical mechanisms of glucose and amino-acid reabsorptions in the tubules. The urines of five cases of typical renal glycosurics, as well as from a normal man who had been given a massive glycosuria by the subcutaneous injection of 20 mg. of phloridzin were examined. In no case was the amino nitrogen output increased.

Table 5. *Sulphur partition of urine of case E.C.*

Date	Total S (mg.)	SO_4-S (mg.)	Ethereal S (mg.)	Neutral S (mg.)	Methionine (mg.)
16 Oct.	717	577 (80%)	62 (9%)	78 (11%)	340

The percentages of the total sulphur are shown in brackets. The methionine contains 73 mg. of sulphur which is 94% of the neutral sulphur. All these figures are within normal limits. All determinations in duplicate.

In the case E.B., of the 4 specimens of urine passed on different days the only one which contained glucose (0·11 g./100 ml.) was the only one with a raised concentration of amino nitrogen (43·7 mg./100 ml.). The trace of glucose in the urine of V.R. was not estimated quantitatively. It is interesting to recall here the intermittent glycosuria found in the original case of Milkman's syndrome (Milkman, 1934).

Was the amino-aciduria the primary cause of the disease? It must first be pointed out that the total output of amino-acids in E.C. is about 10 g./day and therefore corresponds to only a small fraction of his protein intake. There is therefore no question of a general protein deficiency being brought about by the urinary losses. As far as concerns individual amino-acids there is likewise no conspicuous excretion of any particular essential amino-acid, all of which are taken in the diet in much larger quantities than they are being excreted. The only point that will be made is that of the disproportionate loss of hydroxyamino-acids. Serine, in particular, is lost both in the free form and as the peptide 'under-alanine'. Probably 1–2 g./day were put out in the case E.C. Serine, however, is not an essential amino-acid and can presumably be synthesized by the normal body as fast as it may be required. It is interesting, however, to recall the key position which the amino-acid occupies. It can provide the carbon chain for the synthesis of cystine from methionine (du Vigneaud, Kilmer, Rachele & Cohn, 1944). It is a precursor of ethanolamine and hence of choline (Stetten, 1941). It can occur naturally as a phosphoric ester (Lipmann & Levene, 1932; Posternak & Pollaczek, 1940). There is a strong presumption that any of these mechanisms may be defective in the Fanconi syndrome. In addition,

Table 6. *R_F values of amino-acids and other ninhydrin reacting substances on the two-dimensional chromatograms*

($P = R_F$ in phenol. $C = R_F$ value in collidine. All chromatograms run first with phenol then with collidine. The colours are compared against the purple given by valine.)

Substance	P	C	Colour
Aspartic acid	0·17	0·27	Blue
Glutamic acid	0·28	0·29	Purple
Serine	0·35	0·28	Brownish purple
Glycine	0·42	0·22	Reddish purple
Threonine	0·50	0·30	Purple
Alanine	0·62	0·25	Slightly bluish purple
'Under-alanine'	0·59	0·21	Reddish purple
α-Amino-*n*-butyric acid	0·71	0·30	Purple
Histidine	0·77	0·24	Greenish purple
Citrulline	0·66	0·22	Purple
Arginine	0·87	0·13	Purple
Lysine	0·80	0·11	Purple
Valine	0·80	0·34	Purple
Methionine	0·82	0·42	Purple
Leucines	0·85	0·43	Purple
Phenylalanine	0·86	0·44	Blue
Tyrosine	0·63	0·53	Dull brownish purple
Tryptophane	0·77	0·47	Purple
Proline	0·86	0·24	Bright yellow
Hydroxyproline	0·77	0·31	Yellowish brown
'Over-glycine'	0·42	0·35	Purple
Cystine	0·21*	0·11*	Purple
Cysteic acid	0·08	0·35	Blue
Methionine sulphoxide	0·79	0·24	Purple
Methionine sulphone	0·69	0·35	Bluish purple
Dihydroxyphenyl-alanine	0·33*	0·44*	Yellow
Asparagine	0·43	0·21	Olive brown
Glutamine	0·57	0·23	Purple
β-Alanine	0·68	0·21	Purple
Glucosamine	0·20	0·53	Blue
Seryl-glycine	0·53	0·31	Dull yellow turning purple later
Glutathione	0·35*	0·10*	Purple

* These values refer only to one-dimensional chromatograms in the given solvent. It is not usual to see these compounds on two-dimensional chromatograms owing to decomposition.

serine injury to rats (Fishman & Artom, 1945) produces similar urinary changes (proteinuria, glycosuria, occasional amino-aciduria). An attempt to reproduce this on one rat, using chromatographic examination of urines, showed that only serine was present in excessive amounts in the urine.

Sulphur metabolism. Attempts to disclose an error of sulphur metabolism have all met with failure. No cystine deposits were found in the organs of E.C. and V.R. Methionine may be the only amino-acid which is excreted in normal quantities in the urine. The cystine output is at most only slightly raised. Cystine and methionine given in large quantities by mouth were normally metabolized. Methionine sulphoxide has also been found in the urine after the administration of methionine to normal subjects. The methionine saturation curve (Fig. 5) was also within normal limits (Albanese *et al.* 1944).

A very interesting finding after the methionine feeding was the distinct increase in the output of α-amino-*n*-butyric acid. When the serial chromatograms were compared side by side the fact was more convincing than Table 4 would indicate. It has also been seen in a normal adult after methionine had been given by mouth. It appears that a new possible mechanism in the metabolism of methionine has been brought to light, namely, direct desulphurization to α-amino-*n*-butyric acid, such as can be carried out with Raney nickel (author's unpublished work, see also Mozingo, Wolf, Harris & Folkers, 1943). The synthetic *dl*-methionine administered was shown to be chromatographically pure.

Finally it should be noted that when serine had been fed there was no change in sulphur output, nor in the gross distribution of the other amino-acids. A fall in sulphur output might have been expected if the additional serine had been partly diverted to the production of much-needed cystine from methionine.

In conclusion, it is still not possible to attribute the Fanconi syndrome to any single cause. A new form of renal damage involving low thresholds for amino-acids, glucose and phosphorus is proved and will account for all the findings except the cirrhosis of the liver. The possible role of chronic acidosis, especially favoured by Fanconi and by McCune *et al.*, has not been considered here as it was not proved to be present in the case E.C. who was most thoroughly investigated, although the high ammonia coefficient of the urine was suggestive.

SUMMARY

1. The amino-aciduria in three cases of Fanconi syndrome has been investigated with paper chromatograms. One case was more thoroughly investigated from other points of view over a period of 4 months.

2. The amino-aciduria was renal in type, i.e. due to a low kidney threshold for amino-acids. The total output of amino-nitrogen in the urine was related to the output of glucose according to a straight line relationship, although both varied considerably from day to day. It also appeared to be related directly to the fluid balance of the patient.

3. The following free amino-acids were found in the urine of one case (where the 24 hr. output of amino-acid was roughly estimated it is given in parenthesis): cystine, aspartic acid, glutamic acid, serine (0·25 g.), glycine (0·8 g.), threonine (0·5 g.), alanine (1·0 g.), valine (0·5 g.), leucine and/or *iso*-leucine (0·4 g.), methionine (0·34 g.), phenylalanine, tyrosine (0·5 g.), arginine, citrulline, histidine, proline, hydroxyproline and α-amino-*n*-butyric acid. Cysteic acid and methionine sulphoxide were sometimes present but may have been produced by secondary decomposition of cystine and methionine.

4. A ninhydrin-reacting substance 'over-glycine' was found. It is suggested that this is a new amino-acid and its behaviour on the chromatogram suggests that it is of novel composition.

5. A peptide 'under-alanine' containing serine and glycine was also found in large amounts. In one case 0·75 g. at least was excreted daily in the urine. It is believed to be serylglycylglycine, but this has yet to be confirmed by synthesis. Complexes liberating large amounts of aspartic and glutamic acids on hydrolysis, were also present.

6. No error in metabolism of sulphur amino-acids could be shown in one case of Fanconi syndrome specially investigated. After methionine was given by mouth the output of α-amino-*n*-butyric acid increased as if some direct desulphurization had taken place. This is apparently a normal mechanism. There was also a large increase in the output of methionine sulphoxide. This has also been found in normal subjects. Its possible formation in the urine from methionine by secondary oxidation in storage has not been adequately excluded.

7. It has not been possible to prove that the chronic loss of amino-acids in the urine is responsible for the other features of the syndrome.

8. α-Amino-*n*-butyric acid and 'under-alanine' have been found in normal plasma and urine.

9. Developments in the technique of paper chromatography have been devised to enable: (*a*) small quantities of substances in biological fluids to be isolated for further study of their chemical properties; (*b*) the simultaneous detection of histidine, histamine and tyrosine in urines; (*c*) the detection of methionine and cystine without loss from decomposition and without confusion with other amino-acids.

10. In the course of this work it has been shown that most of the common organic and inorganic substances present in urine, and many others

present in pathological conditions, have characteristic movements on the paper chromatograms which are sufficiently specific to constitute a criterion for their recognition. Further, by means of this technique the existence of new compounds may be disclosed.

The writer wishes to thank Prof. H. P. Himsworth for his great interest and encouragement throughout, also Drs D. Hunter and J. H. Sheldon for providing rare pathological material, and Dr R. L. M. Synge for frequent and valuable advice on amino-acid problems. The technical assistance of Mr G. A. Rose is gratefully acknowledged.

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Porphyrinuria in Rats Fed Oxidized-Casein: a Preliminary Communication

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The work described in this paper arose out of attempts by one of us (C.E.D.) to devise a diet sufficiently low in cystine and methionine to produce acute hepatic necrosis in rats (for review see Glynn, Himsworth & Neuberger, 1945) with more success than was possible with natural proteins with a low content of these amino-acids.

Oxidized-casein (Toennies, 1942), produced by the action of hydrogen peroxide on casein dissolved in formic acid, appeared to be eminently suitable for this purpose since Bennett & Toennies (1942) have shown that, when supplemented with tryptophan and methionine, it is nutritionally adequate for the young rat although growth is not quite as good as on natural casein. Food consumption was about 20% lower on the supplemented oxidized-casein diet than on the control diet. This would suggest that no essential amino-acids other than methionine and tryptophan are destroyed by the oxidation procedure. Comparative analyses (Toennies, 1942) of casein and oxidized-casein for threonine, serine, cystine, methionine and tryptophan show that from the latter protein, methionine and tryptophan are completely lacking, cystine is reduced from 0·36% to about 0·16% whilst serine and threonine have not been affected. It was considered that methionine

was oxidized to methionine sulphone which is known to be incapable of replacing methionine for growth purposes in the rat (Bennett, 1941).

Our experience with oxidized-casein confirms the claims of the American authors that by supplementation with tryptophan and methionine it can be made nearly as efficient as casein for growth purposes. Necrosis of the liver did not occur on the presumably cystine- and methionine-deficient diet containing oxidized-casein, but it was noticed that all experimental animals in this group began to pass a somewhat brownish urine in which two absorption bands could be distinguished. These were shown to be due to a porphyrin metal complex and a further study was therefore undertaken to ascertain what relation the porphyrinuria bore to the composition of the diet. Clearly porphyrinuria might have resulted from either a specific deficiency or from the presence of a toxic substance formed during the chemical oxidation of the protein.

The development of porphyrinuria without obvious signs of liver damage or the introduction into the diet of any of the recognized porphyrinurogenic poisons, e.g. lead, sulphonal, etc., is of interest. The identification of the responsible agent might bring to light a relation between pyrrol pigment metabolism and some well-known physiological substance.

In addition, oxidized-casein is now being suggested as a convenient methionine-free protein for biological experiments (Lyman, Moseley, Wood & Hale, 1946) and a warning against its too ready acceptance as a harmless, simplified diet is timely.

MATERIALS AND METHODS

Oxidized-casein

Preparation. The method of Toennies (1942) was modified in several particulars. 800 g. of casein (Grade A, British Drug Houses Ltd.) were added in small lots to 4·8 l. of 98–100% formic acid in an enamelled pan provided with a wooden stirrer, the addition taking about 1 hr. The temperature was 17° throughout. During the addition some lumps formed which mostly disappeared after 0·5 hr. of stirring. 800 ml. of 30% ('90–100 vol.') H_2O_2 were then added and the solution was stirred 1·5 hr., any remaining lumps being broken up as much as possible. 10 l. of water (distilled water used throughout) was then added followed by 5 l. of ice and water. Ammonia (sp.gr. 0·880) was added to bring the pH to about 4 (bromophenol blue greenish), about 4 l. being needed. The temperature was kept below 30° during the neutralization by adding lumps of ice. Finally 5 l. more water were added and the precipitated protein was filtered off and pressed as dry as possible on large Buchner funnels. The cake was returned to the pan and stirred overnight with 20 l. of water. Some lumps were seen at this stage. They were sieved off through gauze and separately worked up by the same method as the main batch. The main batch was again filtered. Filtration was much slower than before. After thorough washing with water on the filter the precipitate was stirred with 2·5 l. of acetone, filtered and dried in thin layers at 100°. The gritty crusts thus obtained were ground and spread out for some days before final storage. Yield 520 g. About 30 g. more were accidentally lost. Yield from lumpy material separately worked, 19 g.

Comparative analyses of casein and oxidized-casein gave:

	Nitrogen (g./100 g. protein)	Methionine (g./100 g. protein)
Original casein	13·4	2·93
Main batch of oxidized-casein	13·50	0·0
Lumpy fraction of oxidized-casein	12·92	0·47

Nitrogen was determined by micro-Kjeldahl; methionine by the method of McCarthy & Sullivan (1941). In view of the methionine analysis, the lumpy fraction was discarded. Two-dimensional chromatography (Consden, Gordon & Martin, 1944) on a hydrolysate of the oxidized-casein was carried out side by side with a hydrolysate of the original casein. The oxidized-casein showed all the amino-acids present in the casein except methionine. Hydroxyproline, which always yields a weak spot, could not be distinguished but may have been present in small amount. Cystine did not appear on either chromatogram. Histidine appeared at first to be a little weaker, but this was not confirmed by comparison against casein hydrolysate using the more specific diazo reaction on the collidine one-dimensional chromatogram (Dent, 1947). Lysine may have been a little weaker. In addition, the oxidized-casein gave a strong spot with the characteristic colour and position due to methionine sulphone, and a weaker but quite definite one for cysteic acid which therefore accounts, in part at least, for the loss of cystine previously reported (for the positions of these spots see Dent, 1947). No methionine sulphoxide was seen. The spots for serine, threonine and tyrosine, which might be expected to be partly oxidized by the H_2O_2, were quite as strong as in the untreated casein.

Methionine sulphoxide and sulphone

These were prepared by the methods of Toennies & Kolb (1939, 1941). The purity of the preparations was confirmed chromatographically.

Rats

Male rats of Wistar strain and of weight approximately 100 g. were used, two on each diet (excepting No. 6 which was tested only on one rat). In the first experiment in which diets 1–4 were compared, litter-mates were employed as shown in Fig. 1. They were housed separately in 10 in. diameter glass funnels provided with a galvanized-iron wire grating; faeces and urine were separated by means of glass bulbs resting in the necks of measuring cylinders. The urines were collected every 2 days, a few drops of toluene being placed in the measuring cylinders at the beginning of each 2-day period. The food was supplied in the form of paste placed in receptacles suspended from the roof of the cage at such a height that the animals were forced to eat directly from them, so preventing the removal of food to the floor of the cage where crumbling occurs with inevitable contamination of the excreta. A similar arrangement has been used in extended nitrogen balance experiments and from the results obtained it is believed that an

average of 95% of the total urinary output is collected. Body weights were recorded every 2 days. Water was provided *ad lib.* The diets were weighed out every day and mixed with water to a stiff paste before feeding. In this way they were invariably eaten without contaminating the excreta. Protein and amino-acid constituents of the various diets are shown in Table 1. Glutamic acid, although easily seen on the chromatogram of oxidized-casein, was added to diet 8 in view of the finding of Borek, Sheiniss, & Waelsh (1946) that it antagonizes the growth-inhibitory action of methionine sulphoxide towards *L. arabinosus*.

supplemented with methionine (3, 8, 9 and 10). In some cases this difficult period was covered by a gradual transition, half casein and half oxidized-casein being fed for a few days.

The amino-acid supplements difficult to dissolve (tryptophan, cystine) were separately weighed, others were added every day to the food as an appropriate solution in the mixing water.

Urine analysis

Partition chromatograms. These were performed by the standard methods as applied to urine by Dent (1946).

Table 1. *Protein and amino-acid constituents of diets*

The amount of each supplement corresponds to that derivable from 0·95 g. casein (Block & Bolling, 1945).

Diets	Casein (g.)	Oxidized-casein (g.)	*l*(−)-Tryptophan (mg.)	*dl*-Methionine (mg.)	*l*(−)-Cystine (mg.)	*dl*-Methionine sulphone (mg.)	*dl*-Methionine sulphoxide (mg.)	*l*(−)-Cysteic acid (mg.)	*l*(+)-Glutamic acid (mg.)	*l*(−)-Histidine (mg.)	*l*(+)-Lysine hydrochloride (mg.)
1. Casein	0·95	—	16	—	—	—	—	—	—	—	—
2. Oxidized-casein	—	0·94	32	—	—	—	—	—	—	—	—
3. Oxidized-casein + methionine	—	0·92	32	27	—	—	—	—	—	—	—
4. Oxidized-casein + cystine	—	0·92	32	—	22	—	—	—	—	—	—
5. Casein + methionine sulphone	0·95	—	16	—	—	33	—	—	—	—	—
6. Casein + methionine sulphoxide	0·95	—	16	—	—	—	30	—	—	—	—
7. Casein + cysteic acid	0·95	—	16	—	—	—	—	4·5	—	—	—
8. Oxidized-casein + methionine + glutamic acid	—	0·95	32	27	—	—	—	—	180	—	—
9. Oxidized-casein + methionine + histidine	—	0·95	32	27	—	—	—	—	—	29	—
10. Oxidized-casein + methionine + lysine	—	0·95	32	27	—	—	—	—	—	—	68

Carbohydrate and salts were given to the extent of 10·5 g./rat/day, of a mixture containing 71% corn starch, 24% sucrose and 5% salt mixture (Glaxo). In addition each rat received 0·5 ml. arachis oil, 2 drops cod-liver oil, 0·5 ml. yeast extract and 0·5 ml. of vitamin solution. The latter contained in 0·5 ml.: aneurin 20 μg., riboflavine 25 μg., pyridoxin 20 μg., calcium pantothenate 100 μg. and choline chloride 12 mg. Twice a week each rat was given 1 drop of vitamin E concentrate containing about 5 mg. of pure tocopherol.

The protein and carbohydrate were separately weighed every day. If any food was left from the previous day the amount was subtracted from the total of carbohydrate mixture to be fed that day. This achieved a relatively even intake of protein and supplements. Appetite was good on all the diets except 2 and 4 (Table 1) so complete eating of the diet was usual. Appetite was usually impaired for a few days on going over to the diets with oxidized-casein

A sample of 25 μl. of urine was taken for each test. In one case, acid hydrolysis was performed by sealing the urine (25 μl.) in a capillary tube with 25 μl. of conc. HCl and immersing in boiling water for 24 hr. The contents were then evaporated to dryness to remove HCl, dissolved in the minimum amount of water and transferred to the paper for chromatography.

Porphyrin determination. Porphyrin was observed to be present in the urines in the form of metal complex. This was no doubt due to unavoidable contamination of the specimens with zinc derived from the wire grid which supported the animals. For accurate quantitative determination of porphyrin content it was therefore necessary to liberate the free porphyrin by means of acid as follows. Urine (5 ml.) was acidified with 0·5 ml. of 20% (w/v) HCl and left for 0·5 hr. at room temperature. Sufficient saturated aqueous potassium acetate was then added to render the mixture neutral to congo red (about 0·5 ml.

required) followed by 0·5 ml. of glacial acetic acid and 10 ml. of a mixture of 9 parts ether with 1 part light petroleum. The mixture was vigorously shaken, the lower layer discarded and the ether phase washed three times with an equal volume of water. The porphyrin was then transferred to 0·25% (w/v) HCl by repeated shakings with about 1·5 ml. quantities, completeness of extraction being controlled by observation in ultra-violet light. The porphyrin content of the acid solution was then determined by fluorimetric comparison with a standard safranine solution (50 mg./l., calibrated against pure coproporphyrin 1) using a Pulfrich step-photometer and an Ilford filter No. 607.

Tests for hepatic insufficiency

Clinical. At the earliest stage of the onset of acute hepatic necrosis the rat becomes very inactive, loses its appetite and stands huddled in a corner of the cage. At this stage, palpation of the abdomen to determine the size of the liver is a most valuable procedure. Marked enlargement of the lobes of the liver is usually easy to detect and has been found only in acute hepatic necrosis. Other signs may also be present, e.g. hunched back, slow deep breathing, yellow staining around the penis, epileptiform convulsions. All these latter however can occur in the absence of acute hepatic necrosis and are therefore misleading. The rectal temperature falls rapidly as the hepatic necrosis develops, being often only about 24° before death. Temperature has not, however, been of much help in diagnosing early liver failure.

Bile pigments in urine. Urobilin was tested for by direct spectroscopy after the addition of a trace of iodine. Bilirubin is detected best by Fouchet's test on 2 ml. of urine. It can readily be modified however for spot tests using only 10–25 μl. of urine. The filter papers used for the test can be kept as permanent records. Generally speaking, bilirubin appears only about one day before death from hepatic necrosis. Rarely, when the disease has been very acute in its clinical onset, bilirubin may be absent throughout, even from the urine in the bladder at post-mortem. On two occasions the Fouchet test has been carried out during attacks followed by recovery. Bilirubin was excreted for about 5 days, this corresponding also to a period of weight loss and loss of appetite.

Amino-aciduria. Normal rat urines usually show a weak band with an *Rf* value of 0·25–0·30 which has been identified as glutamic acid. Glycine (*Rf* 0·36) and alanine (*Rf* 0·57) appear weakly if at all. An excessive output of a wide range of amino-acids, showing easily on the chromatograms, when it occurs, is absolutely diagnostic of acute hepatic necrosis and provides valuable objective confirmation of the condition. It only appears late in the disease and has been absent in one out of about 20 cases, when the onset of the disease was exceptionally acute. One case has been followed during recovery. The excessive output of amino-acids disappeared gradually in about 3 days and closely followed the changes in bilirubin output. Bilirubin however may be present without excessive amino-aciduria, but not vice versa.

Haemoglobin determination

The Haldane method was used, the colour standard being calibrated so that 100% = 13·8 g. Hb/100 ml. Only occasional determinations were performed.

Post-mortem examinations

All animals were examined carefully when killed at the termination of experiments. Liver sections were taken in all cases, other organs being also examined if thought necessary. Negative findings are not reported here.

RESULTS

Growth

Fig. 1 shows the growth curves obtained from the rats on diets 1–4. This experiment can be taken as a biological test for the efficiency of the oxidative removal of methionine from the casein. It is seen

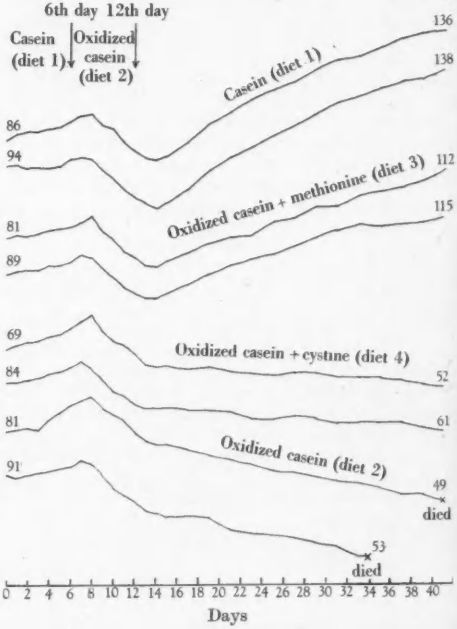

Fig. 1. Growth curves of individual rats on diets 1–4. All upper curves and similarly all lower curves are from litter mates. Body weights (g.) at beginning and end of the experiment are marked on the curves.

that no growth is obtained on the oxidized-casein supplemented only with tryptophan, in fact, weights steadily declined. Addition of methionine, on the other hand, in the quantity which would have been present in the casein, resulted in fair growth but not as good as in the control group on casein alone. These curves agree closely with those of Bennett & Toennies (1942). The mean daily food consumption of each of our rats on the diet containing oxidized-casein + methionine (diet 3) was 10·0 g. as against 11·5 g. for those on casein (diet 1). Addition of

cystine in an amount containing the same total sulphur as the methionine supplement has only a slight effect in reducing the mortality. This confirms that methionine had been completely eliminated since, owing to the methionine-sparing action of cystine, had the elimination been incomplete, slight gains of weight could have been predicted when cystine was fed.

Growth curves are not presented for animals on diets 5–10 since 5, 6 and 7 followed closely diet 1, which also had casein as the source of protein, while 8, 9 and 10 (containing oxidized-casein + methionine) conformed to diet 3.

Failure of oxidized-casein to produce acute hepatic necrosis

Of two rats on oxidized-casein supplemented only with tryptophan (diet 2), one died of pneumonia on the 24th day. Its liver was histologically normal. The other died suddenly on the 42nd day. It was well at 10 a.m. but one hour later it was huddled in a corner of the cage, breathing slowly and jerkily just as does a case of acute hepatic necrosis. It then developed clonic spasms and died at noon. Its liver was not felt on abdominal palpation at any stage. There was complete absence of body fat; the liver and other organs were normal. Its serum specific gravity was 1·0163 (copper sulphate method of Phillips, Van Slyke, Dole, Emerson, Hamilton & Archibald, 1945) which is equivalent to a plasma protein of 3·4 % if the same factor (360) is used as for human blood.

The experiment was repeated with two 200 g. rats since Glynn, Himsworth & Neuberger (1945) showed that with such large animals, acute hepatic necrosis may develop after only 8–22 days on a methionine and cystine deficient synthetic diet. During 50 days on diet 2, the animals lost 55–60 g. in weight but were otherwise normal except for increased porphyrin excretion, the levels of which reached 15 times the normal (see Fig. 2).

One animal was then sacrificed and afforded the following post-mortem findings: Liver—few areas of early necrosis of irregular distribution, probably hydrotropic degeneration. Spleen—slightly deeper iron staining than usual. All other organs including bone marrow, normal. The bones did not fluoresce in ultra-violet light.

The companion to this rat was replaced on diet 1 whereupon he rapidly regained his lost body weight whilst porphyrin excretion promptly fell to near normal limits (see Fig. 2).

Four other rats (approx. 200 g.) were maintained in the same way on diet 2 for 20 days without producing acute hepatic necrosis.

Porphyrin excretion. The urinary porphyrin excretion of our experimental animals is represented by Figs. 2–6. It will be seen that whenever the diet contained oxidized-casein a prompt rise in porphyrin excretion occurred up to something like six times the normal level. No supplementation was able either to prevent or cure this porphyrinuria. When, however, cysteic acid, methionine sulphone or methionine sulphoxide was added to a casein-containing diet, in amount equivalent to the cystine or methionine content of the protein, no increase in porphyrin excretion occurred even after 18 days' administration.

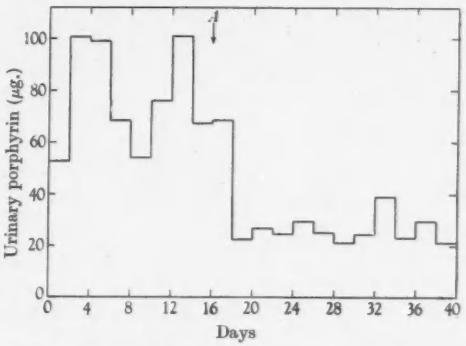

Fig. 2. Urinary porphyrin excretion (2-day collections) of rat on diet (no. 2) containing oxidized-casein. It had already been 5 weeks on this diet prior to commencement of porphyrin determinations. At *A* placed again on basal casein diet (no. 1).

Blood haemoglobin levels

In six instances the level of haemoglobin in the blood was determined before and after the administration of oxidized-casein (diet 2) supplemented only by tryptophan for periods of 19–31 days but no significant change could be detected.

Chromatography of urines on various diets

Casein (no. 1). Normal chromatograms for rats' urines as described in the experimental section have invariably been found.

Oxidized-casein (no. 2). About 30 chromatograms were made at various times on the urines from the six rats on this diet. All were normal except the one rat which died, apparently from starvation. This rat showed slightly excessive amino-acid output during the last week of life. Analysis showed that this was chiefly due to an excess of one amino-acid only, which was identified as methionine sulphone by two-dimensional chromatography. There were also complexes (? peptides) liberating large amounts of amino-acids, especially aspartic and glutamic acids, on HCl hydrolysis. Surprisingly, the urine withdrawn from the bladder at post-mortem contained practically no free amino-acids; a hydrolysis was

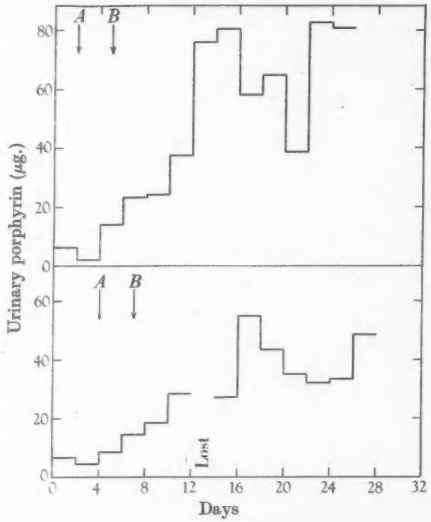

Fig. 3. Urinary porphyrin excretion (2-day collections) of two rats. At *A* basal diet (no. 1) half-replaced by oxidized-casein diet (no. 2). At *B* complete replacement by no. 2.

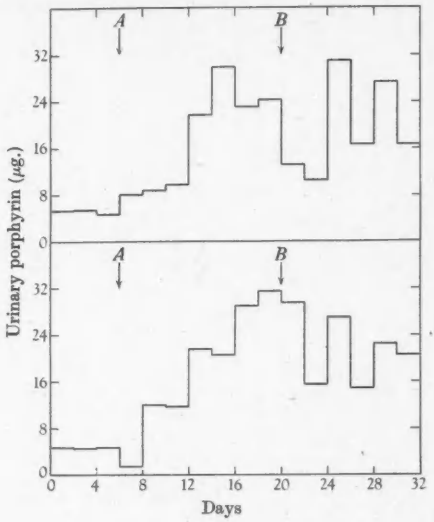

Fig. 5. Urinary porphyrin excretion (2-day collections) of two rats. At *A* basal diet (no. 1) replaced by oxidized-casein plus methionine (diet no. 3). At *B* glutamic acid added (diet no. 8).

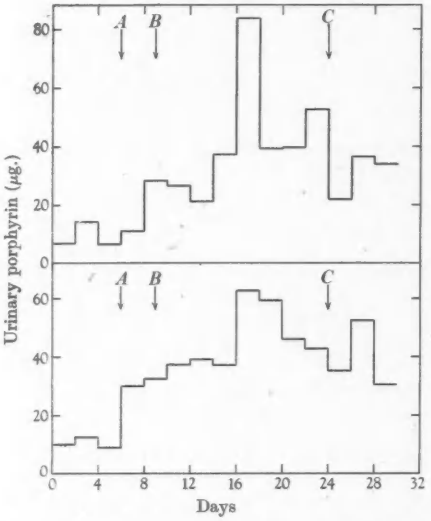

Fig. 4. Urinary porphyrin excretion (2-day collections) of two rats. At *A* basal diet (no. 1) half-replaced by oxidized-casein diet (no. 2). At *B* complete replacement by no. 2. At *C* methionine added (diet no. 3).

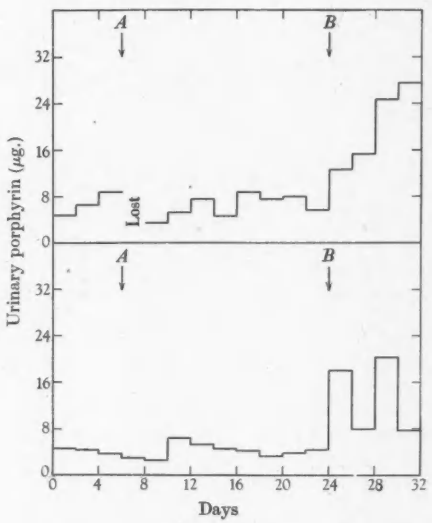

Fig. 6. Urinary porphyrin excretion (2-day collections) of two rats. At *A* basal diet (no. 1) supplemented by methionine sulphone (diet no. 5). At *B* replaced by oxidized-casein plus methionine plus histidine (diet no. 9).

unfortunately not done on this specimen. Methionine sulphone could not be detected in the urine of any of the other five rats on the same diet.

Oxidized-casein plus methionine (*no.* 3). Normal chromatograms throughout.

Casein plus methionine sulphone (*no.* 5). The urine always contained methionine sulphone which, roughly estimated by matching the intensity of the ninhydrin colour, accounted for at least half of the total amount added to the diet. Otherwise the urine was normal.

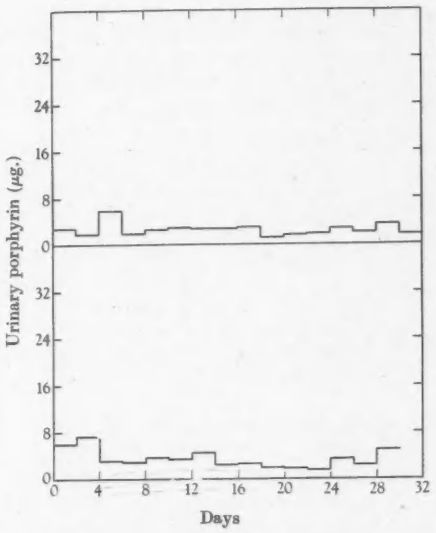

Fig. 7. Urinary porphyrin excretion (2-day collections) of two rats on basal diet (no. 1).

Casein plus methionine sulphoxide (*no.* 6). The urine invariably contained methionine sulphoxide. Rough matching showed that about one-fourth of the ingested sulphoxide was passing out unchanged into the urine. No other amino-acids were excreted in abnormal amount.

Casein plus cysteic acid (*no.* 7). Amino-acid output normal. No cysteic acid seen.

No regular chromatograms were performed on the urines from the rats on the other diets.

DISCUSSION

The experiments recorded have confirmed the fact that, *in vitro*, methionine in casein may be oxidized completely to the sulphone. The protein, apart from simultaneous loss of tryptophan, is not otherwise seriously affected as far as its growth-supporting properties go. This follows from the restoration of a growth rate nearly equal to that of the controls by supplementation of oxidized-casein with tryptophan and methionine.

The oxidized protein has, however, acquired another property, that of causing increased urinary porphyrin excretion, and in seeking to analyze this situation the following possibilities presented themselves: (*a*) the oxidized-casein diet is deficient in some factor, possibly an amino-acid which has been destroyed by the oxidative procedure and the presence of which is essential for normal pigment metabolism; (*b*) oxidized-casein causes injury to the liver of such a nature that porphyrinuria results; (*c*) the oxidation procedure gives rise to a toxic substance, which is porphyrinurogenic.

Possibility (*a*) and in part (*b*) also were tested by feeding as supplement any or all of the amino-acids which analysis and our own chromatographic examination showed to be lacking or diminished in oxidized-casein as compared with the parent protein. Methionine deficiency was of course the first factor suspected since amino-acid diets lacking methionine and cystine have been shown to be not only incapable of supporting growth but also to be productive of acute hepatic necrosis which is massive in type. Porphyrinuria is an almost invariable feature of parenchymatous liver injury (Nesbitt & Snell, 1942, etc.). The inability of methionine to prevent the increase of porphyrin excludes this type of liver injury as an explanation of the porphyrinurogenic action of oxidized-casein. Careful histological examination revealed, with one exception, no abnormalities in the livers of the rats on the diets of unsupplemented oxidized-casein and in this animal individual evidence of functional disturbance of the liver was not forthcoming from the other tests. All that can be said is that if oxidized-casein produces a hepatic lesion, this is not detectable histologically.

A priori support for possibility (*c*) was deduced from the structural similarity between methionine sulphone and sulphonal which is one of the best known of porphyrinurogenic drugs (Salkowsky, 1890–1). Methionine sulphone when fed failed, however, to raise the porphyrin excretion and the sulphoxide was similarly innocuous, as might have been expected from the fact that it can replace methionine in diets (Bennett & Toennies, 1942). The oxidation product of cystine, cysteic acid, similarly failed to raise porphyrin excretion. The products to which tryptophan gives rise during the preparation of oxidized-casein are not known, so they could not be tested. One can only conclude that if a toxic substance is formed during the oxidative procedure, we have failed to identify it, although we would like to draw attention to the possibility that methionine sulphone in peptide linkage may have toxic properties which are not reproduced by feeding the free sulphone itself.

That this latter possibility is not without experimental support is shown by the results of the urine chromatograms. On all the diets in which oxidized-casein was fed, no methionine sulphone was found in the urine (except for one rat, already mentioned, which excreted it only during the last few days before death) although this was shown to be present in the acid hydrolysate of the protein. On the other hand, when methionine sulphone was fed as a supplement to a casein diet (no. 5) it largely overflowed into the urine unchanged. Hence it appears that the body must deal differently with methionine sulphone when it is combined in the form of a peptide, and such a peptide may be the toxic porphyrinurogenic substance.

The failure of acute necrosis of the liver to develop on a diet devoid of methionine and containing very little cystine was unexpected. It is just possible that lesions would develop if the animals were kept on the diet for a longer time. The possibility cannot be excluded, however, that methionine sulphone may act as a protective substance.

Further studies will be undertaken of the phenomena described in this communication. The work has been interrupted at the present stage by the departure of one of us (C.E.D.) on a travelling Fellowship.

SUMMARY

1. It is confirmed that casein treated with hydrogen peroxide ('oxidized-casein'; Toennies, 1942) contains no methionine detectable analytically or by growth experiments.

2. Partition chromatography has revealed the presence in acid-hydrolyzed oxidized-casein of methionine sulphone and cysteic acid but of no other abnormal constituent reacting with ninhydrin.

3. Rats fed a diet including oxidized-casein rapidly develop prophyrinuria.

4. This porphyrinuria is not prevented by supplementation of the diet by methionine, tryptophan, histidine, lysine or glutamic acids alone or in various combinations, although satisfactory growth follows whenever methionine and tryptophan are both added.

5. Acute hepatic necrosis did not occur in rats fed oxidized-casein supplemented only with tryptophan. The livers of those receiving both methionine and tryptophan supplements were invariably normal.

6. The prophyrinuria observed whenever oxidized-casein is present in the diet could not be associated with any histologically detectable liver damage.

7. Neither methionine sulphone nor cysteic acid when added to a casein diet induced porphyinuria.

8. Chromatographic examination of the urines indicates that methionine sulphone is differently metabolized when present in peptide linkage in oxidized-casein than when fed as a supplement.

9. A peptide containing methionine sulphone may be the porphyrinurogenic agent.

We wish to thank Messrs G. A. Rose and J. Evans for technical assistance rendered during the course of this investigation.

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Changes in the Arginase and Alkaline Phosphatase Contents of the Mammary Gland and Liver of the Rat during Pregnancy, Lactation and Mammary Involution

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In a previous paper (Folley & Greenbaum, 1946) we have shown that the lactating mammary gland of the rat exhibits appreciable arginase activity and that in fact it is probably the second richest source of arginase in the body. Since it seemed likely that arginase is concerned in the metabolism of the mammary gland, probably with the synthesis of milk constituents, it was decided to study changes in the arginase content of mammary tissue of the rat during the different phases of growth and function characteristic of pregnancy, lactation and involution. The liver arginase content was also studied at the same time because pregnancy and lactation impose a considerable strain on the metabolism of the body which might be reflected in changes in the liver arginase levels.

The only other enzyme known at present to occur in striking concentration in mammary tissue is the alkaline phosphatase (Folley & Kay, 1935), the role of which in mammary metabolism is not definitely known. This work also provided an opportunity of studying changes in the phosphatase activity of mammary tissue in different states of growth and activity. To furnish an indication of possible changes in the general phosphatase levels of the body, and for comparison with the mammary phosphatase, the kidney phosphatase was also determined.

The evidence presented in this paper indicates that arginase plays a more important role in lactation than has hitherto been suspected.

EXPERIMENTAL

Rats. Hooded Norway rats were used, mated for the first time for this experiment at an age of 3 months. The groups were randomly selected and killed at 5, 10, 15 and 20 days after mating and at 5, 10, 15 and 20 days after parturition (pregnancy in this colony usually lasts 22 days). The pups of two further groups were weaned at 21 days and the mothers killed in the period of mammary involution 3 and 6 days after weaning. In addition, enzyme determinations on liver and kidney tissue from a group of unmated rats were made. The number of rats in each group is given in Table 1. Diet was as described previously (Folley & Cowie, 1944).

Table 1. *Distribution of rats among experimental groups*

	Pregnancy				Lactation				Involution	
Day	5	10	15	20	5	10	15	20	3	6
No. of rats	10	10	10	10	11	11	10	10	7	6

Preparation of tissue homogenates. The general procedure regarding dissection of the tissues was as described previously (Folley & Greenbaum, 1946). An improved tissue homogenizer was used in which the blades revolved at 15,000 r.p.m., and all metallic parts in contact with the fluid were made of stainless steel. Weighed amounts of liver, mammary gland and kidney tissue were dispersed (5 min.) in 9, 19, and 39 times their weight of normal saline respectively, one drop of toluene being added as preservative.

Arginase determination. Pure *l*(+)-arginine monohydrochloride was prepared from gelatine by method 1 of Brand & Sandberg (1932). Arginase activity was determined by a method generally similar to that used previously (Folley & Greenbaum, 1946) but differing in that determinations of the urea produced were replaced by colorimetric (Sakaguchi) estimations of the residual arginine by the method of Albanese & Frankston (1945). This change enabled a reduction to be made in the scale of the estimation, thus effecting a big saving in arginine, and also enabled more estimations to be run. There is the further advantage that one can carry out estimations of fully activated arginase in the presence of added Co^{++} without any danger of error due to decomposition of arginine by urease preparations (Hellerman & Perkins, 1935–6). The enzyme determinations were set up in the same proportions as before but scaled down from 25 ml. to 10 ml. flasks with a corresponding reduction of the other volumes. The procedure was the same up to the point of filtering after inactivation of the enzyme. The filtrate was then diluted 1 in 100 and 5 ml. of this taken for estimation by the method of Albanese & Frankston, with the difference that exactly 2 min. instead of 1 min. were allowed for the action of the hypochlorite. This slight modification was found to make very little difference to the colour produced or to the shape of the standardization curve; it was done for convenience in working through large numbers of samples. The colour was immediately measured in a Miller photoelectric absorptiometer using an Ilford Spectrum green filter (604), corrected for the colour developed by the reagents alone, and

the arginine calculated from a standard curve obtained by the same method. The solutions were kept at 21° throughout. Not all samples of hypochlorite were satisfactory; red label Deosan (Deosan Ltd., London, N. 7) proved suitable and was adopted. Ornithine, in concentration equivalent to 50% hydrolysis of the substrate, was shown not to interfere with colour development. Macpherson (1946), in discussing a similar method of arginine estimation, states, but without details of the concentrations involved, that 'NH_3 gives rise to erratic results and should be removed'. From our previous results (Folley & Greenbaum, 1946) with a method which necessarily involved determinations of preformed NH_3 we might expect that the 5 ml. of diluted filtrate taken for the colorimetric determination would contain not more than 4μg. of free NH_3. In the present experiments our results have never been erratic and moreover we have found that addition of a further 4μg. NH_3 had no effect on the final colour.

Van Slyke & Archibald (1946) are of the opinion that arginase activity should be measured by urea production instead of by residual arginine, because of the possibility of decomposition of arginine, without the production of urea, by enzymes other than arginase. This objection might conceivably apply particularly to our 20 hr. method for mammary gland homogenates. In 16 experiments, however, in which arginase determinations by the 20 hr. method were done on amounts of rat milk equal to the amounts of mammary tissue normally present in our reaction flasks, the decomposition of arginine was negligible, indicating not only the absence from rat milk of arginase but also of other enzymes capable of decomposing arginine without the formation of urea. It seems likely that the same would apply to mammary gland homogenates.

For both liver and mammary gland estimations the same units, '30 min.' and '20 hr.' respectively, and the same calibration curves were used as before. All determinations were begun within 2 or 3 hr. of the preparation of the homogenates.

Alkaline phosphatase. Alkaline phosphatase was determined as before (Folley & Greenbaum, 1946) using a Miller photoelectric absorptiometer with an Ilford Spectrum red filter (608).

Estimation of lactose. Lactose was determined in rat milk and in mammary gland homogenates by an adaptation of the method of Hinton & Macara (1927). In the case of milk a protein-free filtrate was prepared by diluting 0·5 ml. of milk to 25 ml., and to 10 ml. of this adding 0·5 ml. of dialyzed iron, standing for 10 min., diluting to 50 ml. and filtering. Mammary gland homogenates were deproteinized by adding 0·3–0·5 ml. dialyzed iron to 5·0 ml. of homogenate, standing 10 min., diluting to 50 ml., and filtering. This was done immediately after homogenization, as we have evidence which suggests that these homogenates exhibit an increase in iodine uptake on standing at room temperature. To 10 ml. of either filtrate were added 2·5 ml. of 10% KI and 10 ml. of 0·005 N-Chloramine T. The flasks were then stoppered with bungs, which had been steeped in KI, and placed in a constant temperature room at 20° for 90 min. At the end of this time they were removed, 2 ml. of 2 N-HCl were added and the liberated iodine titrated with 0·005 N-thiosulphate. Hinton & Macara give as a factor, 1 ml. 0·04 N-thiosulphate ≡ 6·81 mg. lactose. In this adapted method this figure corresponds to 0·851 mg. lactose/ml. 0·005 N-thiosulphate. In practice this was found to give low results, and as a result of many estimations of pure lactose and of recovery of added lactose from mammary gland homogenates the empirically determined factor of 0·870 was adopted.

Milking of rats. Groups of five rats similar to, but not identical with, those in the main experiment were set aside for milking at 10, 15 and 20 days of lactation. These rats could not be used in the experiment because the handling of the glands in milking interfered with subsequent lactation.

A machine similar to that described by Cox & Mueller (1937) was tried but the yields were insufficient for analysis. The procedure finally adopted was hand milking following an intraperitoneal injection of 5 i.u. oxytocin ('Pituitrin', Parke, Davis and Co.). With suitable manipulation of the glands and teats, sufficient milk for analysis could easily be obtained. In the case of the rats at the 10th day of lactation the milks were collected and analyzed as two composite samples representing two and three rats respectively. The samples from the five rats at the 15th and at the 20th days of lactation respectively were collected and analyzed individually.

RESULTS

Milk content of mammary tissue. In a previous paper (Folley & Greenbaum, 1946) attention was drawn to errors in enzyme determinations on mammary gland tissue due to milk retained in the alveoli and the finest ducts, for the complete removal of which no method is known. Unless the proportion of retained milk can be determined, the true weight of tissue taken for homogenization cannot be ascertained. Since it is likely that at full lactation the proportion of such milk is considerable, enzyme activities expressed per unit weight of moist tissue will be in serious error except in the extremely unlikely event of the enzyme concentration in milk and tissue being equal.

Arginase determinations on milk from rats at the 10th, 15th and 20th days of lactation, both in the presence and absence of Co^{++}, were found to be uniformly negative, and the error would, therefore, be maximal. It would also be serious in the case of phosphatase, which determinations on the rat showed to be in much lower concentration in the milk than in the mammary tissue (Table 2).

It is therefore clear that enzyme determinations on mammary tissue at different stages of pregnancy and lactation, during which the residual milk is certain to vary enormously, would have no meaning unless the milk content of the tissue could be determined and the appropriate corrections applied.

Milk contains two major constituents which are peculiar to it and which are normally found nowhere else in the body, namely casein and lactose, both of which offer possibilities in connexion with the development of a method for determining milk in mammary gland homogenates.

A method based on casein determinations was considered and discarded because of the obvious difficulties of estimating very small quantities of

casein in the presence of other proteins. Lactose has been used for estimating the amount of milk in mammary tissue (Gaines & Sanmann, 1927; Gowen & Tobey, 1927, 1928). To apply this to homogenates of rat mammary tissue it is necessary to show that the reduction measured is due solely to lactose. This was shown by estimating the reducing power of mammary gland homogenates before and after hydrolysis with 2N-HCl, when it was found that the reducing sugar content was doubled, while after incubation of the hydrolysate with bakers' yeast the reducing sugar content was restored to its original level. It is also necessary to know the lactose content of rats' milk. In Table 2 are given the results of determinations of lactose in rats' milk on the 10th, 15th and 20th days of lactation. These results are in essential agreement with those of Cox & Mueller (1937) who used a milking machine.

Table 2. *Lactose and alkaline phosphatase content of rat milk*

Rat no.	Day of lactation	Phosphatase units*/100 ml.	Lactose (g./100 ml.)	Group mean
22,356	10	36·0	2·88	3·00
22,365	10			
22,385	10			
22,390	10	24·0	3·12	
22,190	10			
22,802	15	28·6	3·19	3·37
22,804	15	30·0	3·55	
22,826	15	29·0	3·41	
22,849	15	26·6	3·39	
22,854	15	28·0	3·31	
22,822	20	29·6	3·43	3·18
22,860	20	30·0	3·40	
22,806	20	30·0	3·07	
22,798	20	36·0	3·00	
22,809	20		2·98	

* King & Armstrong (1934) units.

In calculating the milk content of mammary tissue the mean milk lactose content for the appropriate stage of lactation was used when available. Because no values were available for the 5th day of lactation the mean of all values was used here, since a test (variance ratio test) for the homogeneity of variance between the three groups (Snedecor, 1940) showed the values to be homogenous ($F = 2{\cdot}11$, degrees of freedom 2 and 9).* This could probably be done without much error as our results show no evidence of significant changes in the lactose content during lactation. In the case of pregnancy, and perhaps involution, this is less permissible since the composition of fluids withdrawn from the mammary gland of animals pregnant for the first time is very different from normal milk (Asdell, 1925; Woodman & Hammond, 1923). But nevertheless we consider that the application of the milk correction during pregnancy and involution gives a result much nearer the truth than one obtained without it.

Individual lactose estimations were made on all mammary gland homogenates, in each case leading to an estimate of milk content which could be used for correcting the corresponding enzyme determinations to a true moist tissue basis. The group mean percentages of retained milk in mammary tissue at various stages are given in Table 3.

Table 3. *Milk content* (%) *of mammary gland tissue*

	Pregnancy			
Day	5	10	15	20
Mean retained milk (%)	9·61	25·66	30·14	26·45
	Lactation			
Day	5	10	15	20
Mean retained milk (%)	41·01	45·15	51·04	54·20
	Involution			
Day		3	6	
Mean retained milk (%)		34·26	24·75	

Gross and corrected group mean mammary gland weights for the six abdominal glands are shown in Fig. 1. The uncorrected mammary gland weights show a continuous and rapid increase to the end of lactation followed by a precipitous drop after weaning, while the corrected values increase to about the middle of pregnancy and thereafter remain sensibly constant until the third day of involution. A test for the homogeneity of variance showed that values for the four stages of lactation together with the last stage of pregnancy were homogenous ($F = 1{\cdot}10$, degrees of freedom 4 and 47). These results provide independent biochemical evidence in support of the currently accepted view, based on histological and cytological evidence (see Turner, 1939), according to which mammary gland hyperplasia is held to be substantially complete by mid-pregnancy and any further apparent growth is hypertrophy due to distention of the alveolar cells, and the alveoli themselves, with secretion. It will be seen that at the end of lactation the correction factor is of the order of 2×, so that determinations made without such corrections are in serious error. Moreover the factor varies between 1·1× and 2·2× at different stages of pregnancy and lactation, thus emphasizing the particular necessity for applying a milk correction in an investigation of this kind. In

* Throughout this paper, where F values are given, the number of degrees of freedom corresponding to the greater mean square is given first.

the case of phosphatase, which occurs in low concentration in milk, a further correction for the amount of phosphatase in retained milk was applied although this was small and did not appreciably alter the results.

With regard to this method for correcting for the milk content of mammary tissue it must be remembered that lactose may occur in secretory droplets in the cytoplasm of the alveolar cells in a different concentration from that of the milk which can be drawn from the gland for analysis and further that the lactose content of the milk retained in the alveoli themselves may also differ slightly from the latter. However, it seems probable that these effects will be far too small to invalidate the correction.

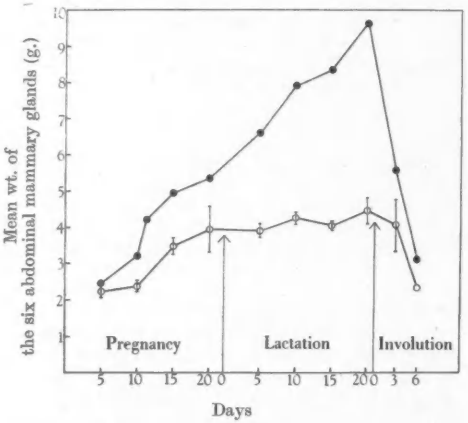

Fig. 1. Mean weights of the six abdominal mammary glands for groups of rats during pregnancy, lactation and mammary involution. The black dots represent the gross mammary gland weights; the circles represent values corrected for milk retained in the tissue. The vertical lines passing through the circles represent twice the S.E.M. When no vertical line is given on the corrected weight curve the S.E.M. is too small to show.

Variations in arginase activity of the mammary tissue. Changes in mammary arginase/g. of moist tissue are shown in Fig. 2. During the period of mammary growth, i.e. early pregnancy, there is relatively little increase in arginase content nor is there any appreciable increase up to the 5th day of lactation. But after the 5th day of lactation there begins a rapid increase in arginase activity which continues uninterruptedly to the end of lactation at which point it is some six times the value at the beginning of lactation. After weaning there is a precipitous fall back to the level characteristic of early pregnancy. A curve showing the total arginase activity of the six abdominal glands is not given

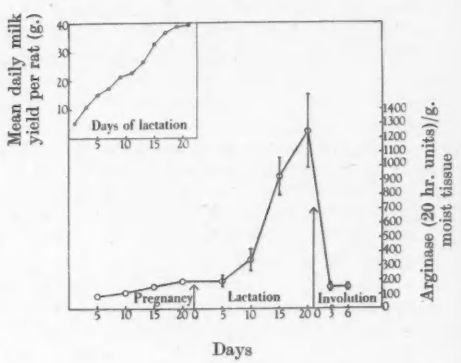

Fig. 2. Variations in mammary gland arginase. The vertical lines are equal to twice the S.E.M. When no vertical line is given, the S.E.M. is too small to show. The inset represents the lactation curve of the rat, constructed from the data of Brody & Nisbet (1938).

since the true weight of the glands remains sensibly constant throughout lactation (Fig. 1) and the curve is obviously of the same form.

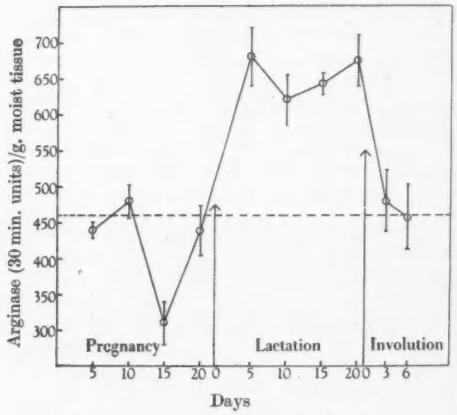

Fig. 3. Variations in the liver arginase/g. moist tissue. The horizontal broken line is the non-pregnant control level. The vertical lines are equal to twice the S.E.M.

Variations in liver arginase activity. The variations in liver arginase/g. of moist tissue are shown in Fig. 3. The liver arginase/g. in pregnancy remains constant and not significantly different from the non-

pregnant control level except the values for the 15th day which showed a marked fall. A test for the homogeneity of variance among the pregnant groups shows that the drop at the 15th day is significant ($F = 7{\cdot}99$, degrees of freedom 3 and 36). No explanation for this can be given at the present time.

The most important point brought out by the figure is the sharp rise following parturition. The values obtained throughout lactation do not differ significantly among themselves ($F = 0{\cdot}73$, degrees of freedom 37 and 3) and must be regarded as constant, but the general lactation level is very significantly higher than the level at the 5th and 10th days of gestation ($F = 12{\cdot}1$, degrees of freedom 5 and 56). Following weaning there was a sharp drop back to the level characteristic of early pregnancy.

Our data provide some suggestion of a regular increase in liver weight in late pregnancy and lactation, over and above that due to general body growth (Fig. 4). This tends to enhance the increase in

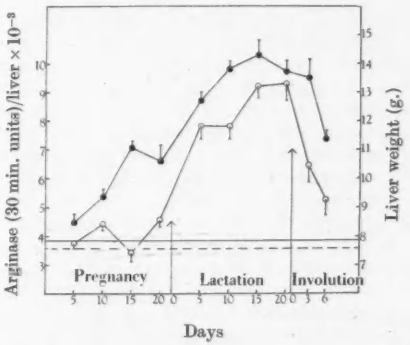

Fig. 4. Variations in the total liver arginase and the liver weight. Circles represent total liver arginase; black dots represent liver weight. The intact horizontal line is the non-pregnant control level for liver weight. The broken horizontal line is the non-pregnant control lever for total liver arginase. In order to avoid confusion each vertical line represents the S.E.M. in one direction only.

liver arginase following parturition when calculated on the basis of the whole liver, and there is some indication that the total liver arginase rises slowly over the whole lactation period.

The enhanced liver arginase activity (per g. moist tissue) characteristic of lactation stands in contrast to the state of affairs during pregnancy where the values were not significantly different from the non-pregnant rats, except in the case of the 15th day of pregnancy where the value was smaller. It is interesting that the increased strain on the maternal metabolism due to the needs of the growing foetuses is not of a nature which requires an increase in the liver arginase activity, whereas lactation evidently necessitates such an increase.

Variations in the mammary gland phosphatase. In contradistinction to the mammary gland arginase, the mammary gland phosphatase (Fig. 5) increases steadily throughout pregnancy and reaches a maximal value soon after parturition, which is maintained constant throughout lactation (confirmed by a test for homogeneity of variance for the four lactation values giving a value of $F = 1{\cdot}27$, degrees of freedom 3 and 38). Again there is a sharp decrease after weaning but it is not so precipitous as in the case of arginase, i.e. the involution level is still

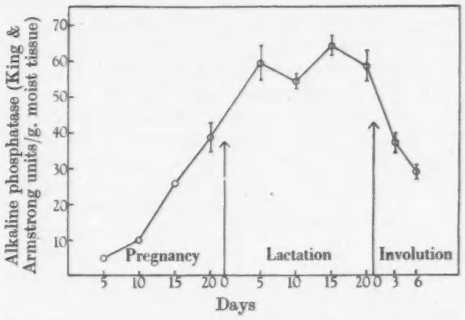

Fig. 5. Variations in mammary gland alkaline phosphatase. The vertical lines are equal to twice the S.E.M. Where no vertical line is given the S.E.M. is too small to show.

some three times that of early pregnancy. Kidney phosphatase/g. of moist tissue (not shown in the figure), used as a reference standard, remained substantially constant throughout.

DISCUSSION

Increase in mammary gland arginase content at mid-lactation. It is not clear whether the dramatic rise in mammary arginase at mid-lactation is a process initiated between the 5th and 10th days of lactation or is of the nature of an autocatalytic increase in response to a stimulus operating at some time during pregnancy. Inspection of Fig. 2, indeed, suggests that most of the curve up to the end of lactation could be fitted by a logistic equation corresponding to a unimolecular autocatalytic chemical reaction. If so, this would agree with the second alternative. It seems desirable, however, in the present state of knowledge, to discuss our results on the basis of the former, less complicated, assumption, as it seems possible that while the relatively

small but nevertheless statistically significant increase between the 5th and 20th days of pregnancy may be connected with the phase of mammary growth (and Edlbacher & Merz (1927) have suggested a correlation between cellular multiplication and tissue arginase), the increase occurring during lactation must certainly have no connexion with mammary growth, which has long since ceased.

This dramatic rise in mammary gland arginase which takes place in mid-lactation can only mean that arginase occupies a key position in the metabolism of the lactating, as contrasted with the non-lactating mammary gland. The nature of its function in lactation is a point of considerable interest and importance. The only well-established role of arginase in mammalian metabolism is as a participant in the production of urea through the 'ornithine cycle'. It may be noted, however, that this mechanism for the formation of urea has not found universal acceptance (see Bach, Crook & Williamson, 1944), and there is a possibility that urea formation may not involve arginase at all. Even if the ornithine cycle provides the true and only mechanism for urea production in the mammal, a quantitative parallelism between liver arginase content and the level of urea production does not necessarily follow. As far as we know no such parallelism has been conclusively demonstrated. However, there is a decrease in liver arginase after adrenalectomy (Fraenkel-Conrat, Simpson & Evans, 1943; Folley & Greenbaum, 1946), a procedure which presumably results in decreased deamination of amino-acids, though it is not at present known whether or not the primary cause of this effect is a reduction in food intake which results from adrenalectomy. But on the other hand liver tumours may show a high arginase content coupled with a disability to synthesize urea (Neber, quoted by Edlbacher & Koller, 1934; Greenstein, 1943). Nevertheless, in our opinion, the consensus of evidence would appear to favour the view that arginase participates in urea production in some such scheme as that postulated by Krebs & Henseleit (1932), and as a reasonable corollary, increased deamination and urea formation might well demand an increased tissue arginase.

If the mammary gland arginase is, like that of the liver, concerned with deamination and urea production, with gluconeogenesis in fact, it should be possible to demonstrate (*a*) an uptake of amino-acids by the active mammary gland and (*b*) an output of urea. Arterio-venous studies, in fact, have uniformly shown a small but definite uptake of amino-acids by the lactating gland of the cow (Blackwood, 1932; Shaw & Petersen, 1938*a*, *b*; Bottomley & Folley, unpublished results) and the goat (Lintzel, 1934; Graham, Jnr., 1937–8; Graham, Jnr., Peterson, Houchin & Turner, 1937–8; Reineke, Peterson, Houchin & Turner, 1939) but not by the non-lactating bovine udder (Blackwood, 1932). An appreciable output of urea by the udder of the lactating goat has been reported by Graham, Jnr., Houchin & Turner (1937) and Reineke *et al.* (1939).

On the basis of balance experiments on the lactating ruminant mammary gland it has been claimed (Graham, Jnr., 1937–8; Shaw & Petersen, 1938*a*, *b*; Reineke *et al.* 1939) that the uptake of amino-acids is quite insufficient to account for the observed synthesis of casein. It therefore seems probable that blood amino-acids absorbed by the mammary gland are not directly concerned in the synthesis of casein, a conclusion supported by the failure of Reineke, Williamson & Turner (1941) to detect an uptake of amino-acids by the udder of the fasting goat which nevertheless continued to secrete some milk, but may be utilized for a process analogous to gluconeogenesis. Indeed Graham, Jnr. (1937–8) speculated on the possibility that lactose precursors might arise from the deamination of amino-acids by the active mammary gland without providing any direct evidence in support of this supposition. The present results seem to strengthen the idea that the deamination of amino-acids, absorbed from the blood stream as such or perhaps in the form of protein (e.g. see Reineke *et al.* 1941), with the formation of substances concerned in carbohydrate metabolism, and also, according to modern views (see Stadie, 1945), in fat metabolism as well, is an important element in the metabolism of the lactating mammary gland.

The increase in mammary gland arginase at mid-lactation must clearly be considered in relation to our finding that the liver arginase content increases immediately after parturition and remains elevated throughout lactation.

The mammary gland is an exceptional organ, situated outside the body but receiving its metabolites from the common blood stream. At the height of its activity it shows a remarkable capacity for the synthesis of its specific protein, fat and sugar. In the rat, unlike the ruminant in which the milk yield rises to a peak relatively early in the lactation period and thereafter steadily falls off, such data as are available indicate a steady increase in milk yield over the whole lactation period of 21 days. The measurement of milk yield in the rat is an extremely difficult operation and the only data known to us are those of Brody & Nisbet (1938) obtained by a complicated, and not entirely unobjectionable, method. A mean lactation curve calculated from some of their data is shown in the inset of Fig. 2. It seems possible that if intermediates derived from the deamination of amino-acids are indeed essential substrates for milk synthesis, the liver is able during early lactation when, in the rat, milk secretion is proceeding at a relatively low level, to satisfy both

the normal demands of the body and the additional ones of the mammary gland, the added burden due to lactation being reflected in an increase in its content of arginase. But when, in mid-lactation, the rate of milk synthesis has passed a given point, the liver can no longer cope with the increased demand for deaminized residues and the mammary gland is forced to realize, to a striking degree, its potentialities, evidenced by the presence of relatively small amounts of arginase in the non-lactating gland, for gluconeogenesis. On this view the mammary gland in full lactation may be regarded as in some respects analogous to the liver of an animal in a state of 'overproduction' (e.g. pancreatic diabetes; phloridzin diabetes), in that in the effort to maintain a high output of sugar, amino-acids are increasingly used as sugar precursors. In this connexion it would be of interest to investigate whether the induction of diabetes is accompanied by a rise in liver arginase. Takehara (1938) has indeed reported increased liver arginase levels during fasting in the dog, which were restored to normal by feeding carbohydrate but not by a diet of fat and protein.

Other possible roles of mammary gland arginase may be briefly discussed. Edlbacher & Merz (1927) have postulated a correlation between tissue growth and arginase content. Though our results show some evidence of a slight increase in mammary gland arginase in pregnancy, during the earlier part of which most of the mammary growth occurs, and to this extent may be said to agree with the views of Edlbacher & Merz, the large increase characteristic of mid-lactation cannot have any connexion with mammary growth since most authorities agree (e.g. Weatherford, 1929) on the rarity of mitosis during lactation. A second possibility arises from the suggestion put forward by Edlbacher and his collaborators (see Edlbacher, 1938) that cellular arginase may be concerned with protein synthesis in the cell. During lactation the mammary gland is a site of active protein synthesis and on this view the mammary gland arginase might well be concerned with the synthesis of casein. From the present evidence as discussed above, however, it would seem more likely that the mammary arginase is primarily concerned with deamination processes.

Changes in the mammary gland alkaline phosphatase. The changes in alkaline phosphatase content of mammary tissue are strikingly different from the arginase changes in that the former are confined to the period of pregnancy. The form of the curve expressing the changes in phosphatase content of the mammary gland is generally similar to that showing the increase in weight of the mammary gland tissue. It is perhaps unwise to conclude from this that the role of the alkaline phosphatase in the mammary gland is concerned merely with the growth phase which necessarily involves synthesis of nucleoproteins, as suggested by Moog (1946), since the mammary phosphatase level remains high throughout lactation. The additional possibilities that during the secretory phase it may be concerned with the synthesis of casein or with dephosphorylation processes connected with carbohydrate metabolism, or even with the capture of blood sugar molecules by the mammary gland cells, must not be overlooked.

Phosphatase activity of mammary gland as compared with that of kidney. In our previous paper (Folley & Greenbaum, 1946) we reported an approximate value of 7 for the kidney phosphatase/mammary gland phosphatase ratio in the rat on the 21st day of lactation. This mean value referred to six animals only and no correction could then be made for the milk content of the mammary tissue. The present results which are more extensive and which have been corrected for the milk content of the mammary tissue permit a more accurate comparison of the phosphatase activity of the mammary gland with that of the kidney, as representing one of the richest sources of alkaline phosphatase in the mammalian body. Since the mammary phosphatase values for the four groups of lactating rats were found to be drawn from the same statistical population (see above) these can be considered for this purpose as one group of 42 animals. The same applies to the corresponding kidney phosphatase values since in point of fact all groups investigated including the non-pregnant non-lactating controls were also statistically homogeneous.

The mean corrected mammary gland phosphatase activity for the 42 animals was 57·8 King & Armstrong units/g. moist tissue, the corresponding mean value for the kidneys of the same animals being 163·5 King & Armstrong units/g. moist tissue. The weighted mean kidney/mammary gland ratio was $2{\cdot}88 \pm 0{\cdot}095$. It may be of interest to compare these values with those of the non-lactating mammary gland as represented by the group of ten rats at the 5th day of pregnancy. For these ten animals the mean corrected mammary gland phosphatase activity was 4·96 King & Armstrong units/g. moist tissue and the mean kidney phosphatase activity 149·7 King & Armstrong units/g. moist tissue. The weighted mean kidney/mammary gland ratio was $34{\cdot}24 \pm 4{\cdot}34$.

Unfortunately our present data do not allow us to make a re-evaluation, on the basis of values corrected for retained milk, of our previous estimate of the liver/mammary gland arginase activity ratio for the lactating rat, because experiments at present under way have revealed considerable difficulties in the determination of the ratio between our 30 min. and 20 hr. arginase units. This ratio must be known in order to compare mammary gland and liver arginase activities under the conditions of these experiments.

An accurate comparison between the arginase activities of mammary gland and liver tissue is of considerable importance and the question is being investigated further.

SUMMARY

1. Changes in the mammary gland and liver arginase, and the mammary gland and kidney alkaline phosphatase, have been studied in pregnancy, lactation and mammary involution in the rat.

2. A method is described for the estimation of retained milk in mammary gland tissue enabling mammary gland enzyme determinations to be corrected to a true moist tissue basis.

3. The liver arginase content (per g. moist tissue) during pregnancy and after weaning is indistinguishable from that of unmated controls except at the 15th day of pregnancy when significantly lower values are observed. Over the whole lactation period the liver arginase is significantly increased above these values.

4. The mammary gland arginase increases slowly during pregnancy and early lactation but undergoes a dramatic increase between the 5th and 10th days of lactation. It reaches a maximum at the 20th day of lactation and after weaning falls precipitously to the level of early pregnancy.

5. The alkaline phosphatase content of the mammary gland increases rapidly during pregnancy, reaching a maximum at parturition, and remaining at the same level throughout lactation; it decreases after weaning to a level intermediate between those of lactation and early pregnancy. The alkaline phosphatase content of kidney tissue remains constant throughout pregnancy, lactation and mammary involution.

6. The results are believed to indicate that the mammary gland arginase is concerned with the deamination of amino-acids by the mammary gland, and that the increased arginase content during late lactation in the rat is due to an increased requirement of deaminized residues for the synthesis of milk constituents.

7. The results are consistent with the view that the alkaline phosphatase of the mammary gland may be concerned with the synthesis of nucleoproteins during the growth phase and possibly with the synthesis of casein and the capture of blood sugar molecules during lactation.

8. During lactation in the rat, kidney tissue contains on the average 2·9 times as much alkaline phosphatase as mammary gland tissue. During the mammary growth phase, i.e. in early pregnancy, the kidney/mammary gland ratio is approximately 34.

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Absorption of Carotene from Carrots in Man and the Use of the Quantitative Chromic Oxide Indicator Method in the Absorption Experiments

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The findings of various investigators concerning the absorption of carotene from vegetables in man differ greatly. Wilson, Das Gupta & Ahmad (1937) state that only 10–20 % of the carotene from carrots or spinach is excreted in the faeces. Van Eekelen & Pannevis (1938) found 99 % of the carotene from carrots and 95 % of the carotene from spinach in the faeces. Kreula & Virtanen (1939) and Virtanen & Kreula (1941) observed a carotene excretion varying between 64 and 99 %, and generally amounting to about 90 %. The absorption from very finely grated carrots seemed to be more favourable than from carrots merely masticated. With (1940) observed with purees of tomatoes, carrots, spinach, and carrot meal, excretion values of from 40 to 70 %. Extensive experiments carried out by the Vitamin A Sub-Committee of the Accessory Food Factors Committee (Lister Institute and Medical Research Council, 1945) showed a carotene excretion of about 75 % from carrots and of about 60 % from spinach. If the carotene is dissolved in fat, a noticeably greater part of it is absorbed. Thus With (1940) observed in man excretion values of 25–70 % while the above mentioned Committee found 28 %.

METHODS

Quantitative chromic oxide method

If carotene is determined in the usual way in the faeces, the excreta must be collected quantitatively during several days and stored, frequently for a considerable time, before analysis.

In this respect the quantitative chromic oxide indicator method adopted by Edin (1926) offers noticeable advantages. This method is based on the fact that chromic oxide is not absorbed from the alimentary canal of animals and man, and is entirely excreted in the faeces. The excretion of carotene can be calculated from the proportions of carotene and chromic oxide in the food and in the faeces, and the quantitative collection of faeces is not necessary. In order to find out the excretion of carotene contained in a food, the excretion of carotene derived from the basal diet must also be taken into consideration. This can be determined in a preliminary test. If the basal diet remains unchanged in separate experiments, the basal excretion value determined for each subject may be used in a number of absorption experiments. The net excretion is determined by subtracting from the total carotene excreted during the experimental period (calculated for instance/g. of Cr_2O_3) the carotene correspondingly excreted during the basal period.

Two experiments were made to find out the suitability of the chromic oxide indicator method for the investigation of carotene absorption in man. In one a mixture of carrot and olive oil was used as a source of carotene, in the other, grated carrot alone. In the first experiment the excretion was determined simultaneously by the method involving the collection of faeces and by the quantitative chromic oxide indicator method; in the second only the latter method was employed.

Carotene determinations

In both experiments carotene was determined by the phase separation method (Virtanen & Kreula, 1941), and in the first one also by the chromatographic method according to With (1940). For phase separation, saponification was carried out under nitrogen. The procedure now used in the phase separation method differed from that in the method

previously employed in that the light petroleum (b.p. 50–80°) extract was separated in a suction flask (Jena filter 17G3) and the residue transferred to a porcelain mortar in which it was ground with 20–30 ml. of light petroleum. The manipulation was repeated twice. In general the solvent was colourless after the first treatment. The ethanolic portion of the liquid from the suction flask was combined with the ethanolic extract and the light petroleum was diluted to a fixed volume of either 100 or 150 ml.

From 10 to 15 g. carrot were used for the carotene determination and a total of 50 g. faeces with a requisite amount of sand (Virtanen & Kreula, 1941). As a rule determinations were made in duplicate except those on faeces in Exp. 1 after the first and third periods. The carotene content of the extracts obtained by the phase separation procedure was determined chromatographically.

Chromatographic analysis

The chromatographic analyses were done according to With (1940). The Al_2O_3 used was a Merck preparation standardized according to Brockmann. For analysis 5 ml. of the carotene extract in light petroleum were put through a column 10 × 70 mm. Light petroleum (b.p. 50–80°), generally a quantity of 15 ml., was also used for developing the chromatogram, the carotene passing through, and the bile pigments remaining on the column. Colorimetric determinations were done with a Pulfrich step photometer with the filter S47 and a 5 cm. cell. The percentage excretion of carotene was calculated either directly from the extinction values or from the corresponding weights of carotene calculated according to Halden & Unger (1936).

Determination of chromium

Chromium was determined in 2 g. of dry matter according to Andersen (1934), using nickel crucibles.

ing food was eaten. The basal diet during the experiment consisted of ½ l. skim milk, ½ l. buttermilk or soured skim milk, thin hard rye bread (Swedish 'knäckebröd'), porridge cooked of rye meal, *ad libitum*, 35 g. sugar, and 50 mg. vitamin C per day per person. Water, salt, and saccharine were taken *ad libitum*.

First experiment

Experimental periods. The experiment was divided into three periods. The first period without any carotene-containing foods lasted 5 days. The faeces of the last 2 days were collected for the analysis. The second period during which carrot and Cr_2O_3 were taken began on the sixth day of the experiment and lasted 3 days. In the third period which consisted of the next 2 days only the basal diet was consumed. In both these periods the faeces were collected quantitatively in ¾ l. jars.

Additions. Raw carrot grated with a grater was mixed with olive oil and the mixture passed fifteen times through a meat grinder. The proportions were 580 g. of grated carrot and 116 g. of olive oil. At the beginning of the second period each subject consumed in one day 100 g. of the carotene-olive oil mixture containing 4·0 g. of chromic oxide (3·938 g. Cr_2O_3 by analysis). B took all at one meal, while H and K ate it in two portions.

Results. Table 1 shows the excretion of dry matter and Cr_2O_3 in the faeces. On the average 97·5 % of the chromic oxide consumed was excreted in the faeces. In calculating absorption this percentage was taken as 100.

Table 1. *First experiment. Excretion of dry matter and* Cr_2O_3

Subject	Experimental period	Faeces: Fresh weight (g.)	Faeces: Dry matter (%)	Faeces: Dry matter (g.)	Cr_2O_3 excretion: Total (g.)	Cr_2O_3 excretion: Percentage of dry matter	Cr_2O_3 excretion: Percentage of consumed Cr_2O_3
B	I	663	20·14	133·5	0	—	—
	II	1179	20·80	245·2	3·801	1·55	96·69
	III	791	21·52	170·2	0	—	—
H	I	169	23·50	39·72	0	—	—
	II	697	24·08	167·84	3·627	2·165	95·45*
	III	354	25·84	91·47	0·115	0·125	
K	I	303	25·32	76·72	0	—	—
	II	833	24·59	204·83	3·848	1·879	100·35
	III	418	22·77	95·20	0·104	0·052	

* The low value is due to an analytical error in the collecting of faeces.

EXPERIMENTAL

Test subjects and diet

Three subjects took part in the experiments: H, a woman, aged 23 years and B and K, men aged 31 and 39 years respectively. For 3 days before the start of the actual experiment no carotene-contain-

The carotene content of faeces obtained by the phase separation method and by chromatographic analysis is given in Table 2.

The carrot and olive oil mixture used in this experiment was calculated by the phase separation method to contain 7·20 mg. of carotene/100 g. mixture. For a solution containing 1·0 g. of the material/100 ml. the extinction E was 0·784. By

Table 2. *First experiment. Carotene content of faeces and carotene excretion expressed as mg./g.* Cr_2O_3

				Carotene excretion					
						Chromatographic analysis			
				Phase separation method		Of extracts obtained by the phase separation		Of extracts obtained by With's method	
Subject	Experimental period	Total quantity of faeces (g.)	Dry matter (%)	Total quantity (mg.)	mg./g. Cr_2O_3	Total quantity (mg.)	mg./g. Cr_2O_3	Total quantity (mg.)	mg./g. Cr_2O_3
B	I	163	20·14	0·322	—	0·093	—	—	—
	II	1179	20·80	5·423	1·426	4·893	1·287	4·657	1·225
	III	791	21·52	0·593	—	0·127	—	—	—
H	I	169	23·50	0·118	—	0·037	—	—	—
	II	697	24·08	4·080	1·125	3·764	1·038	3·555	0·980
	III	354	25·84	0·425	—	0·446	—	—	—
K	I	303	25·32	0·212	—	0·055	—	—	—
	II	833	24·59	4·540	1·179	—	—	4·207	1·093
	III	418	22·77	0·259	—	0·121	—	—	—

extracting the pigments soluble in light petroleum directly from the ethanolic extract, an E value of 0·80 was obtained. When the 85 % acetone extraction method adopted by With (1940) was employed, the corresponding extinction was $E = 0·83$. For the chromatographic analysis the column was 10×70 mm. of Al_2O_3, and 5·0–6·25 ml. of carotene extract were used. As the solubility of the carotenoids in 85 % acetone seemed rather slight, an additional determination was made using at first a 96 % methanol extraction followed by extraction with light petroleum. This gave an E of 0·79. According to the chromatographic analysis the carrot material was calculated to contain 7·42 mg. of carotene/100 g. The ratio of carotene to chromic oxide in the food was thus 1·828 mg./g. Cr_2O_3 if the phase separation method was employed, and 1·884 mg./g. Cr_2O_3 if the chromatographic method was used.

The values shown in Table 3 were obtained by calculating the excretion percentages on the basis of both the method involving collection of faeces and of the chromic oxide indicator method. For subjects H and K the carotene values of the third experimental period were included in the total carotene excretion, and the values of the first experimental period were used for calculating the basal excretion. The mean values of the first and third experimental periods were used for the same purpose for subject B, and the carotene excretion was determined from the dry matter values of the second period. In calculations based on the chromic oxide indicator method the carotene and Cr_2O_3 values of the second period were used. The basal excretion was determined for H and K from values obtained in the first period, and for B from the mean for the first and third experimental periods. This could be done, since the basal excretion of carotene formed only a small portion of the total excretion during the experiment and the error probably caused in this way did not essentially affect the final result.

Calculations based on extinction values or on weights of carotene gave similar excretion percentages. As Table 3 shows, there were only slight differences between the excretion percentages calculated by the different methods. The carotene values determined from the faeces according to With (1940), with ether as solvent, were lower than the values obtained by other methods, hence also the carotene excretion percentages were lower.

Table 3. *First experiment. Percentage excretion of carotene calculated on the basis of the faeces collection method and of the* Cr_2O_3 *indicator method*

	On the basis of the collection method			On the basis of the Cr_2O_3 indicator method		
		Chromatographic analysis			Chromatographic analysis	
Subject	Phase separation method	With's method	Extraction with light petroleum	Phase separation method	With's method	Extraction with light petroleum
B	65·3	60·4	63·6	67·6	62·6	65·9
H	52·0	50·7	53·6	54·2	49·8	52·9
K	55·4	55·5	—	56·7	56·1	—

Absorption of carotene dissolved in oil. The carotene content of the oil separated by centrifugation from the carrot and olive oil mixture was found by phase separation to be 27·8 mg./100 g. The total quantity of oil consumed, 16·67 g., thus contained 4·64 mg. of carotene. On the assumption that in this experiment, as in the next one, 10 % of the carotene in the carrot itself was absorbed, and taking for the excretion percentages of subjects B, H and K respectively 65, 52 and 56, the following absorption values were obtained for the carotene dissolved in oil: B, 48·8 %; H, 69 %; and K, 62·8 %.

Second experiment

The second absorption experiment was done by the chromic oxide indicator method with finely grated raw carrots without oil. The same subjects were used. In addition to the basal diet each person took daily 100 g. of carrot and 3 g. of chromic oxide, evenly divided between three meals. The carotene content of the grated carrot, determined by phase separation, was 6·69 mg./100 g. and the carotene content of the food was thus 2·27 mg./g. Cr_2O_3.

Each voiding of faeces was analyzed separately for dry matter, Cr_2O_3 content (in 2 g. dry matter), and carotene content, the last determined in 50 g. samples of faeces by phase separation. The results of the analyses are given in Table 4.

is 2·06 mg./g. Cr_2O_3 and of subject K, 1·982 mg./g. Cr_2O_3. The ratio of carrot carotene to chromic oxide in the food being 2·27 mg./g., the following percentage excretion values are obtained: for B,

$$\frac{2{\cdot}079}{2{\cdot}27} \times 100 = 91{\cdot}6;$$

for H, 90·8; and for K, 87·3. The excretion value was calculated for H from the determinations on 18 and 19 April. The values of 17 April were erroneous since the subject had eaten plenty of vegetable-containing food before the test.

DISCUSSION

The reliability of determinations of carotene absorption depends on many circumstances. The choice of the right method for the determination of carotene is of great importance for the final result. If the source of carotene used in the experiment is analyzed by a different method than the faeces, the results are obviously not quite comparable. Some experiments performed by With (1940) suffer from this weakness. Carotene values obtained for faeces by phase separation are generally somewhat too high. This is, however, partly corrected by the higher basal excretion values. The chromatographic analysis gives the most reliable carotene values if no losses occur during the extraction of carotene and if a 'fluid chromatogram' is used.

Table 4. *Second experiment. Chromic oxide and carotene contents of the faeces*

(First dose of carotene-Cr_2O_3 was taken on 15 April.)

Date of excretion	Weight of fresh faeces (g.)			Dry matter (%)			Cr_2O_3 (% of the dry matter)			Carotene (mg./g. Cr_2O_3)		
	B	H	K	B	H	K	B	H	K	B	H	K
16. iv	*c.* 200	—	—	21·40	—	—	3·266	—	—	2·117	—	—
17. iv, *a*	344	70	140	20·49	32·35	26·91	2·790	1·162*	2·898	2·222	3·530*	2·179
17. iv, *b*	219	76	219	20·11	28·73	22·52	3·297	2·080*	3·354	1·975	2·466*	2·021
18. iv	585	320	737	18·34	26·00	19·71	2·808	4·750	2·445	2·310	2·140	—
19. iv	457	73	321	19·40	29·10	21·61	2·716	4·821	3·142	2·260	2·100	2·017
					Average		2·975	4·786	2·960	2·177	2·120	2·072

* Not included in the average.

B, Subject B; H, Subject H; K, Subject K.

Calculation of the net excretion of carotene. The net carotene excretion, for instance with subject B, calculated from the basal values of the previous experiment (Tables 1, 2), is 2·079 mg./g. Cr_2O_3, the total excretion being 2·177 mg./g. Cr_2O_3. The Cr_2O_3 percentage being 2·975 (Table 4), 1 g. of Cr_2O_3 is equivalent to 33·61 g. dry matter and to 32·61 g. chromic oxide-free dry matter, and the carotene amount excreted within this quantity is 0·098 mg. (0·30 mg./100 g. dry matter). Hence the net excretion is $2{\cdot}177 - 0{\cdot}098 = 2{\cdot}079$ mg./g. Cr_2O_3. The corresponding net excretion value of subject H

The absorption of carotene dissolved in oil seems to depend on the dose of oil as indicated by the results of the first experiment. The absorption percentage of B was 50, of H and K, 70 and 63 respectively. Determination of fat in the faeces showed, however, no apparent correlation between fat and carotene absorption.

The results show that the quantitative chromic oxide indicator method is very suitable for the investigation of carotene absorption. It gives values completely in accordance with those obtained by the method based on the quantitative collection of

faeces and by its use absorption experiments are much simplified and shortened. The faeces also need not be collected quantitatively which facilitates the treatment of the experimental material.

SUMMARY

1. The absorption of carotene was examined with three human subjects. As a source of carotene, raw finely grated carrot or a mixture of grated carrot and olive oil was used.

2. The percentage excretions were calculated by the method of quantitative collection of faeces, and also by the quantitative chromic oxide indicator method which gave the same results.

3. Of the different methods for determination of carotene, chromatographic analysis was found to be the most reliable.

4. The excretion of carotene in human beings on a fat-free diet amounted to about 90 % when finely grated carrot was consumed and to about 30–50 % when carotene dissolved in oil was taken. When the latter was taken in two portions, the excretion was 30–37 % of the carotene dissolved, when taken at one time, the excretion was 50 %.

The above work on the absorption of carotene belongs to the investigations of NJF's Kommitté för foderkvalitet (Scandinavian Society for Agronomy, Committee on the Quality of Fodder). I wish to express my sincere gratitude to the Committee for granting the means for this work.

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The Effect of Sodium Alginate on the Absorption of Calcium

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Alginic acid behaves as a monobasic acid, forming salts with a number of positive ions and its strength is comparable with that of monochloroacetic acid. Water soluble salts of Li, Na, K, Cl, Rb, NH_4 and Mg are known, and these form viscous colloidal solutions which on evaporation leave transparent films; whereas the Cu, Zn, Ag and Ni salts are insoluble in water but soluble in ammonia. The addition of Ca, Ba, Hg, Pb or Bi ions to a soluble salt results in precipitation, for salts of these metals are insoluble in water and ammonia. If these ions are released slowly from sparingly soluble substances, e.g. calcium citrate and dicalcium phosphate, continuous gels can be formed in the cold.

Because alginic acid and its salts are able to withstand any degree of heating without loss of stabilizing value or development of off flavours, this chemically reactive colloid has diverse application in the food industry to replace gelatin, agar agar, Irish moss, pectin, gum tragacanth, starch and other colloids. Solutions can be kept sterile by pasteurization for 1 hr. at 50°, or by treating with preservatives.

Alginic acid is sold in the form of its sodium salt under the trade name of Manucol. Manucol I contains 15–20 % water and a 1 % solution has a viscosity of 15–30 centistokes at 25°. Manucol IV also contains 10–20 % water but a 1 % solution has a viscosity of 80–150 centistokes at 25°. These two products are used widely as stabilizing agents in ice-cream, reconstituted and artificial creams, chocolate milk suspensions, marsh-mallows, fruit squashes, and as thickening agents in custards, jams, marmalades, sauces, soups and water jellies.

Owing to their increased application in the manufacture of foods, it was considered necessary to investigate the possibility of alginic acid or alginates interfering with calcium absorption in man. Calcium alginate is stable to a pH of 3·9, below which the calcium ion is released and the

insoluble alginic acid is formed; but it is not known if conditions in the alimentary tract are favourable for calcium release.

EXPERIMENTAL

Experimental subjects. A calcium balance experiment was done with the following six normal healthy adults.

No.	Sex	Body weight (lb.)	Occupation
1	Male	139	University research worker
2	Female	105	University research worker
3	Female	115	University research worker
4	Female	110	University student
5	Female	129	University lecturer
6	Female	131	University lecturer

Plan of experiment. The subjects were given 4 preliminary days to adjust themselves to the diet. There were two analytical periods of 7 days, the control period and the experimental period when the sodium alginate was taken by mouth. Sodium alginate (Manucol I, 8 g. dry weight) was taken in three approximately equal amounts on each day of the experimental period. It was mixed with some item in the meal such as porridge, soup, meat or dessert. This quantity was thought to be in excess of the amount any person would be likely to consume in manufactured products in one day. On the assumption that the equivalent weight for alginic acid is 176, 8 g. of sodium alginate would correspond to 0·809 g. of calcium.

Diet. All foods were served in the Department of Biochemistry. Lunch and the evening meal were supplied by the Royal Melbourne Hospital diet kitchen. The meals were similar to those provided for the nursing staff except that milk was restricted and cheese was not included. The following is an example of a typical menu.

Breakfast	Lunch	Evening meal
Oatmeal porridge	Roast lamb and mint sauce and gravy	Scotch broth
Toasted white bread	Roast potato	Cold brawn
Butter	Diced carrots	Salad
Marmalade	Peach sponge and custard	Toasted white bread
Coffee		Butter
		Jam
		Coffee

Mid-afternoon—lemon (juice) or other raw fruit

To minimize the interference of phytic acid with the absorption of calcium (McCance & Widdowson, 1942–3; Widdowson, 1941), the bread was made of white flour and the oatmeal porridge was soaked overnight. Foods listed by Kohman (1939) as having a high oxalate content, e.g. spinach, New Zealand spinach, beet-tops and rhubarb, were avoided, as not only is the calcium in these foods unavailable, but the oxalate content is sufficient to make unavailable considerable amounts of the calcium in other foods (Fairbank & Mitchell, 1938; Speirs, 1939).

The quantity of foods eaten, except milk, was left to individual choice and calculation from food tables compiled by the Institute of Anatomy, Canberra, showed that all subjects received adequate calories, protein and at least 0·8 g. calcium daily. This amount is recommended by the Food and Nutrition Board of National Research Council of America (1945) as adequate for an adult. To ensure that each person received sufficient vitamin C, 20 ml. of lemon juice were given every second day.

In addition to the milk used in food preparation, each person was allowed 400 ml. daily. The volume was measured accurately every morning and set aside in individual containers. All milk and the water rinsings of the containers had to be consumed.

Sampling of diet. Each person had a plate, cup, etc. which was weighed and numbered. All food except bread, jam, butter, sugar and salt was weighed directly on to the plate just prior to the meal and placed in an electric oven to reheat it before it was eaten. The methods suggested by McCance & Widdowson (1942–3) were adopted to ensure that the sample was representative of the whole. The servings were given in accordance with the subject's appetite and all waste such as bones and extra fat was removed to avoid weighing back after the meal. The weighings were done on a pan balance which weighed to the nearest gram. A fifth of the amount consumed by each person was weighed to the nearest 0·1 g. and placed in glass specimen jars large enough to hold all the samples for the analytical period. The jars were closely covered and no preservative was necessary.

The butter, jam, salt and sugar were weighed out at the beginning of the week and again at the end, and a fifth of the amount eaten was added to the food samples. Each person had a loaf of bread which was weighed and sampled at the beginning of the week and the remainder then had to be used.

All liquids except milk were sampled for analysis by putting aside an equal volume in a liquid sample jar. With milk, a tenth of the quantity allowed was measured by burette and kept in a bottle under toluene for analysis. Tap water was used throughout and, despite its low calcium content of 2·1 mg./l., it was considered advisable to include it for analysis.

Sampling of excreta. Toluene was used as a preservative for the urine. The bladder was emptied after breakfast on the first day and this specimen was discarded. All other specimens were collected, including the one after breakfast on the eighth day.

Carmine (0·4 g.) was taken before breakfast on the first and eighth day to tag the faeces. Glass specimen jars were used to collect all faeces for the week and no preservative was used. Collection began with the specimen which was stained by the dye taken on the first day, and continued until the second dose of carmine appeared. The portion which was stained by the carmine taken on the eighth day

Table 1. *The effect of sodium alginate on the retention of calcium*

(For the subjects marked A, the control period was the first analytical period and for those marked B, the second analytical period.)

Subject no.	Period	Metabolism of calcium: Intake (mg./day)	Output (mg./day): Urine	Output (mg./day): Faeces	Balance (mg./day)	Absorption (%)
1A	Control	938	258	658	+ 22	30
	Experimental	921	244	624	+ 53	32
2B	Control	883	234	636	+ 13	28
	Experimental	1002	220	566	+216	43
3A	Control	854	108	737	+ 9	14
	Experimental	947	160	713	+ 74	25
4A	Control	—	—	—	—	—
	Experimental	930	273	585	+ 73	37
5B	Control	940	125	832	− 17	14
	Experimental	876	124	771	− 19	12
6B	Control	921	186	681	+ 54	26
	Experimental	962	164	735	+ 62	24

was excluded. The diet was continued until the last collection was completed. No laxatives were permitted or required during the experimental period.

Analytical technique. The analytical arrangements for drying, ashing, and the preparation of the solutions for assay have been described by McCance & Widdowson (1942–3). In the first week the following quantities were taken for analysis: 1/100th of the weight of food and liquids including milk, 100 ml. urine and 50 g. faeces. In the second week smaller portions were found to be more convenient. The calcium was determined by the micro-method described by McCance & Shipp (1933).

RESULTS

The results of the balance experiment are given in Table 1. McCance & Widdowson (1942–3) consider that balances within ±25 mg./day are not significantly positive or negative.

The results of this experiment suggest that sodium alginate does not interfere with the absorption of calcium in normal healthy adults.

SUMMARY

A calcium balance experiment was done with six healthy adults. The experimental periods were of 7 days—a control period and a test period during which 8 g. sodium alginate were taken daily by mouth. No evidence was obtained to suggest that sodium alginate interferes with calcium absorption from a normal varied diet.

This investigation was carried out during the tenure by one of us (F. B. R.) of a grant for research in Nutrition from the Australian National Health and Medical Research Council. We desire to acknowledge the gift of Manucol I from the Imperial Chemical Industries of Australia and New Zealand Ltd., the help given by the Superintendent of the Royal Melbourne Hospital in making available the services of the diet kitchen, and the willing co-operation of our associates, R. D., K. L., J. D., and B. W., in submitting to the discipline of the experiment.

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The Effect of Temperature on the Fermentation of *d*-Mannose by Yeast

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Of all the hexoses found in nature or prepared in the laboratory only *d*-glucose, *d*-fructose and *d*-mannose are fermented by yeasts. On solution in water the crystalline forms of these hexoses exhibit mutarotation resulting in an equilibrium mixture of various tautomeric modifications. Since within the physiological range of temperature this reversible interconversion proceeds quickly, no conclusion can be drawn from experiments of the usual type as to which of the various isomerides present actually undergoes fermentation. In order to obtain more detailed information in this respect fermentations have been carried out at 0°, at which temperature the rate of mutarotation of the hexose added in the solid form is greatly reduced. Under these conditions α-*d*-glucopyranose is found to be freely fermented (Gottschalk, 1943*a*); the same holds for β-*d*-glucopyranose (unpublished). If β-*d*-fructopyranose is added to a yeast suspension at 0°, the rate of fermentation is only a small fraction of that obtained with α-*d*-glucopyranose, and is independent of yeast concentration. In fact the rate is almost identical with that at which the β-*d*-fructopyranose mutarotates into *d*-fructofuranose (Gottschalk, 1943*a*). When yeast at 0° acts upon the equilibrium mixture of *d*-fructose containing the pyranose and furanose forms, a limited amount of the keto-sugar is consumed (Gottschalk, 1943*b*). *d*-Fructofuranose, set free from sucrose or raffinose by saccharase, is readily fermented at 0° (Gottschalk, 1945). From these results it is concluded that only the furanose form of *d*-fructose is suitable for fermentation, thus substantiating a hypothesis first advanced by Hopkins (1931). In the present paper the effect of temperature, from 0 to 25°, on the fermentation of α- and β-*d*-mannopyranose by living yeast and yeast extract is investigated.

MATERIALS AND METHODS

Materials. The experiments were carried out with baker's yeast (Effront), brewer's mild ale top yeast (*Sacch. cerevisiae*) and with brewer's bottom yeast (*Sacch. carlsbergensis*). The brewer's yeast was collected at the Carlton Brewery, Melbourne, and treated as described previously (Gottschalk, 1946). Brewer's bottom yeast was washed and pressed by means of a hand press, the yeast was then dried for 48 hr. either at 27–28° or at 30°. To prepare the Lebedev extract, 20 g. of the dried and thoroughly ground yeast were well mixed with 60 ml. of boiled yeast juice freshly prepared from the bottom yeast in the usual manner (cf. Neuberg & Gottschalk, 1925). After the addition of 100 mg. *d*-glucose the mixture, contained in a round-bottom flask, was immersed in a water-bath at 25° for 4 hr. or at 35° for 2 hr.; every 10 min. the flask was shaken. Following the extraction, the suspension was cooled and centrifuged. The supernatant liquid was immediately used for the experiment.

The acid-soluble inorganic phosphate concentration of the yeast extract thus prepared is on an average 0·040 M, i.e. about the same as that of the intact cell (cf. Gottschalk, 1946). The inorganic phosphate determinations were carried out by the method of Kuttner & Cohen (1927).

The α-*d*-glucopyranose and β-*d*-mannopyranose used in the experiments were pure, anhydrous substances; α-*d*-mannopyranose was prepared from β-*d*-mannopyranose by the method of Levene (1924). The specific rotations of the hexoses (in water; $c=3$, $l=2$) are as follows:

α-*d*-glucopyranose $[\alpha]_D^{20°}$: initial value (extrapolated), +108·5°; after 28 hr., +52·4°.

α-*d*-mannopyranose $[\alpha]_D^{20°}$: initial value (extrapolated), +26·5°; after 24 hr., +14·4°.

β-*d*-mannopyranose $[\alpha]_D^{20°}$: initial value (extrapolated), −16·0°; after 24 hr., +14·2°.

Technique. The experiments were performed at 0° in a refrigerated laboratory (Gottschalk, 1945). Fresh baker's yeast (12 g.) was suspended in 17·6 ml. of 0·1 M-KH_2PO_4, cooled to 0° and equilibrated with nitrogen (freed from oxygen). Immediately before its addition to the suspension the sugar, contained in a small vessel, was dissolved in 0·5 ml. of ice water and the vessel rinsed once with 0·5 ml. of ice water. If an equilibrium mixture of the hexose was to be used in the experiment, the sugar solution was incubated at 25° for 24 hr. and cooled to 0° several hours before the experiment. A corresponding assay without sugar served as control. At the end of the experiment the yeast was spun down at 0° and the supernatant liquid analyzed in triplicate for unfermented sugar by the Hagedorn-Jensen method, using a microburette. It was confirmed that the reducing powers of *d*-glucose and of *d*-mannose are equal (cf. Sobotka & Reiner, 1930).

In the experiments performed at 10 and 25° the rate of fermentation was determined with the apparatus described by Gottschalk & Rawlinson (1942). Constancy of temperature at 10° (±0·25) was obtained by regulating the room temperature to 10–11° and making the final adjustment inside the thermostat with trays of shaven ice and solid CO_2.

When living cells were used, 1–6 g. of yeast, depending on the temperature of the experiment, were suspended in 0·05 M-KH_2PO_4 (total vol. 50·0 ml.); 20·0 ml. of this suspension (pH 4·5) were pipetted into the bulb of the apparatus. The liquid phase was equilibrated against nitrogen.

The sugar, dissolved either freshly or 24 hr. previously in 1·0–1·4 ml. water, was then added. Readings were taken at 5 min. intervals and the volumes obtained reduced to N.T.P. The highest rate of CO_2 production (ml. CO_2/5 min.) during the first 35 min. of each experiment was taken as the fermentation rate for the given substrate concentration.

In experiments with cell-free preparations 10·0 ml. of the yeast extract (pH = 5·96) plus 0·25 ml. toluene were used in each assay. After equilibration of the system the sugar solution and 0·30 ml. of 1% acetaldehyde were added and readings taken every 4 min. There was no CO_2 production in the control without sugar.

The dry weight of the yeast was determined as before (Gottschalk, 1943*a*). In calculating the sugar concentration inside the living cell it was assumed that 96% of the total water of the cell is available as solvent to an added solute (cf. Hill, 1930). The volume of the yeast cells, as ascertained by haematocrit, did not change appreciably during the experiment. The pH measurements were made with the glass electrode.

RESULTS

It may be seen from Table 1 that at 0° and in low concentration, α-*d*-mannopyranose, β-*d*-mannopyranose and the equilibrium mixture of *d*-mannose are fermented at a minute rate only. After a fermentation period of 24 min., about 76% of the added mannose may be recovered from the suspending fluid; even if it is assumed that at the end of the experiment the concentration of *d*-mannose inside the yeast cell is not more than 0·001 M, only 2·5 mg. of *d*-mannose are consumed, compared with 16 mg. of α-*d*-glucopyranose under otherwise similar conditions.

Table 1. *Anaerobic consumption of d-mannopyranose and d-glucopyranose by baker's yeast at 0°*

(Substrate concentration: 0·005 M. Fermentation period: 24 min.)

Substrate	Hexose added (mg.)	Hexose recovered from suspending fluid (mg.)	Hexose disappearing from suspending fluid (as % of that added)
β-*d*-Mannopyranose	16·2	12·6	22·2
α-*d*-Mannopyranose	16·4	12·2	25·6
d-Mannose (equil. mixt.)	16·8	12·8	23·8
α-*d*-Glucopyranose	16·4	0	100

The results summarized in Table 2 demonstrate the effect of various substrate concentrations on the fermentation rate of *d*-mannose (equilibr.) and of

Table 2. *Rates of fermentation of d-mannose and d-glucose by brewer's yeasts at 10° and at 25° with various substrate concentrations*

No.	Yeast: Type	Yeast: Amount (g.)	Substrate concentration (M)	Fermentation rate (ml. CO_2/5 min.): With *d*-mannose*	Fermentation rate (ml. CO_2/5 min.): With *d*-glucose*	Ratio of fermentation rates $\left(\frac{\text{mannose}}{\text{glucose}}\right)$
			(*a*) Temperature 10°			
1*a*†	Top (ale)	6	0·026	0·47	1·04	0·45
2*a*	,,	6	0·130	1·17	1·61	0·73
3*a*	,,	6	0·013	0·36	0·90	0·40
3*b*	,,	6	0·026	0·49	1·10	0·45
3*c*	,,	6	0·106	1·03	1·51	0·68
3*d*	,,	6	0·252	1·36	1·59	0·86
4*a*	Bottom	5	0·026	0·42	0·96	0·44
5*a*	,,	5	0·026	0·40	0·93	0·43
5*b*	,,	5	0·252	1·11	1·28	0·87
6*a*	,,	5	0·026	0·41	0·94	0·44
6*b*	,,	5	0·130	0·95	1·27	0·75
			(*b*) Temperature 25°			
1*b*†	Top (ale)	1	0·013	0·79	1·00	0·79
1*c*	,,	1	0·026	1·04	1·19	0·87
1*d*	,,	1	0·106	1·31	1·39	0·94
2*b*	,,	1	0·130	1·93	2·06	0·94
7*a*	,,	1	0·026	1·25	1·64	0·76
7*b*	,,	1	0·130	1·77	1·92	0·92
4*b*	Bottom	1	0·026	0·92	1·15	0·80
8*a*	,,	1	0·026	0·76	1·02	0·75
8*b*	,,	1	0·252	1·26	1·46	0·86
6*c*	,,	1	0·026	0·87	1·05	0·83
6*d*	,,	1	0·130	1·19	1·38	0·86
6*e*	,,	1	0·252	1·26	1·45	0·87

* The sugar solution was allowed to equilibrate before the experiment.

† Experiments denoted by the same number were carried out within a few hours with portions of the same yeast suspension freshly prepared for the series.

d-glucose (equilibr.) at 10 and at 25°. From the experimental data the ratio

$$\frac{\text{fermentation rate of } d\text{-mannose}}{\text{fermentation rate of } d\text{-glucose}}$$

has been calculated, and its variation with substrate concentration and temperature is shown in the last column of Table 2. At 10° and in low concentration, *d*-mannose is fermented by brewer's yeast at less than half the rate of *d*-glucose; at higher concentration the difference between the fermentation rates of the two epimeric hexoses becomes much smaller. At 25° and low concentration, the fermentation rate of *d*-mannose may be only 20–25 % less than that of *d*-glucose, whereas in higher concentration, *d*-mannose is fermented 10–15 % slower than *d*-glucose. The results obtained with brewer's top and bottom yeasts are very similar.

Table 3. *Rate of fermentation of α- and β-d-mannopyranose by brewer's top yeast at* 10°

(Initial substrate conc. 0·026 M.)

Substrate	Rate of fermentation (ml. CO_2/5 min.) Exp. 1	Exp. 2
β-*d*-Mannopyranose	0·28	0·39
α-*d*-Mannopyranose	0·33	0·45
α-*d*-Glucopyranose	0·88	1·00

In Table 3 the fermentation rates of α-*d*-mannopyranose, β-*d*-mannopyranose and α-*d*-glucopyranose by brewer's yeast at 10° are compared.

Table 3, 6·1 and 6·9 mg. respectively of *d*-mannose were consumed within the first 15 min. during which period 14·0 and 7·0 mg. respectively of β-*d*-mannopyranose and α-*d*-mannopyranose were interconverted to isomerides (cf. Hudson & Sawyer, 1917). The alternative conclusion that only a single *d*-mannose modification, e.g. the α-pyranose form

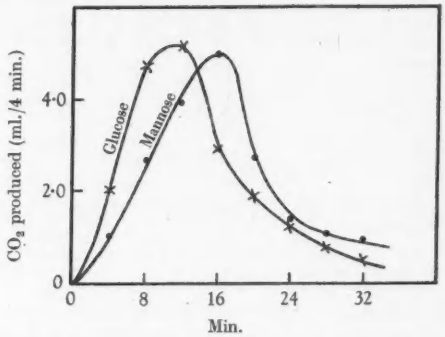

Fig. 1. Fermentation rates of *d*-mannose and *d*-glucose by yeast extract plotted against time. 25°. Inorganic phosphate 0·04 M. Substrate conc. 0·074 M.

or *d*-mannofuranose, which latter contributes a small proportion to the equilibrium mixture, might be suitable for fermentation can be ruled out by considering the substrate concentrations. A comparison of the fermentation rates of α- and β-*d*-mannopyranose seems to indicate a slight preference for the α-form by baker's and brewer's yeasts

Table 4. *Maximum rates of fermentation of d-mannose and of d-glucose by extract of brewer's bottom yeast at* 10° *and at* 25°

Preparation and extraction of the dried yeast	Temp. of exp.	Substrate conc. (M)	Max. rate of fermentation (ml. CO_2/4 min.) *d*-mannose	*d*-glucose	Ratio of fermentation rates $\left(\frac{\text{mannose}}{\text{glucose}}\right)$
Dried at 28° (48 hr.), extracted at 25° (4 hr.)	10°	0·025	1·08	1·08	1·00
	10°	0·025	1·02	1·07	0·95
	25°	0·050	5·00	5·14	0·97
Dried at 30° (48 hr.), extracted at 35° (2 hr.)	25°	0·050	2·95	4·62	0·64

When fermentation is effected by cell-free yeast extract prepared at temperatures not exceeding 28°, the marked difference in the fermentation rates of *d*-mannose and *d*-glucose, observed when these sugars at low concentration are fermented at 10° by living yeast, disappears completely (Table 4). (See also Fig. 1.)

DISCUSSION

From the results obtained at 10° with living yeast, it may be concluded that both α- and β-*d*-mannopyranose are fermentable without preliminary interconversion by mutarotation. Thus, in Exp. 2, (Tables 1 and 3). In these features *d*-mannose parallels *d*-glucose.

A marked difference, however, between mannose and glucose in their behaviour towards yeast is revealed by the finding (Table 2) that at 25° the ratio $\frac{\text{fermentation rate of } d\text{-mannose}}{\text{fermentation rate of } d\text{-glucose}}$ varies only to a small extent with substrate concentration, whereas at 10° this ratio is greatly reduced with decreasing substrate concentration. This unusual dependency on substrate concentration of the temperature coefficient of mannose fermentation by yeast is

clearly demonstrated by Exp. 6, Table 2. From the figures recorded it may be calculated that the ratios of the velocity of fermentation at 25° to that at 10° (V_{25}/V_{10}) are 6·2 and 10·6 for 0·130 M and 0·026 M-*d*-mannose respectively; the corresponding figures for *d*-glucose are 5·5 and 5·6. The further observation that the striking difference in the fermentation rates of *d*-mannose and *d*-glucose in low concentration at 10° is found only with intact yeast cells and not with yeast extract strongly suggests that the cell membrane is responsible for this phenomenon. It would appear that *d*-mannose on entering the cell, i.e. at the water-lipoid interface, meets a higher energy barrier than does *d*-glucose. With high substrate concentrations at low temperatures differences between *d*-mannose and *d*-glucose with respect to the number of molecules possessing the minimum kinetic energy necessary to overcome the corresponding energy barriers at the interface (activated molecules) do not greatly affect the ratio of their fermentation rates, because in each case the number of molecules penetrating the cell membrane will be sufficient to saturate or nearly to saturate the enzyme catalyzing the initial fermentation reaction. With low substrate concentrations, however, differences between *d*-mannose and *d*-glucose in regard to the number of activated molecules will have a marked effect on the ratio, since under these conditions the rate of the initiating reaction is very sensitive to a change in substrate concentration. Increase in temperature from 10 to 25° greatly increases the proportion of activated molecules. This accounts satisfactorily for the experimental result that at 25° the ratio

$$\frac{\text{fermentation rate of } d\text{-mannose}}{\text{fermentation rate of } d\text{-glucose}}$$

varies much less with substrate concentration over the whole range tested than it does at 10°. The finding that at 0° and 0·005 M substrate concentration the fermentation rate of mannose is only a fraction of that of glucose (Table 1) is in good agreement with this interpretation.

In this connexion attention may be drawn to an observation made by Leibowitz & Hestrin (1939). In experiments with living brewer's yeast the ratio of the fermentation rate of methyl-α-glucoside to that of *d*-glucose was found to be 0·24 at 35°, 0·033 at 25° and $1/\infty$ at 4°. Maltose, on the other hand, was fermented at practically the same rate as glucose at the various temperatures. At the substrate concentration used (10 %) the fermentation rate of methyl-α-glucoside was still markedly dependent on concentration, while those of glucose and maltose had already reached their maxima. With maceration juice of brewer's yeast, however, the maximum fermentation rates of glucose, maltose and methyl-α-glucoside were identical both at 28° and at 4°, even at 2 % substrate concentration. Leibowitz & Hestrin supposed that the fermentation paths of methyl-α-glucoside and maltose are different, the former being fermented only after hydrolysis by maltase, to which they ascribed an extremely high temperature coefficient, and the latter being fermented directly. Taken with our results this work would suggest that the extraordinarily high temperature coefficient of the fermentation of methyl-α-glucoside by living yeast is due to the high energy barrier encountered by the glucoside at the water-lipid interface (cf. Kozawa, 1914). The observations of Leibowitz & Hestrin and in the present paper are in harmony with the finding of Jacobs, Glassman & Parpart (1935) that the temperature coefficients of haemolysis of mammalian erythrocytes are high in the presence of non-electrolytes which only slowly penetrate the cell membrane.

Equality of the maximum fermentation rates of *d*-mannose and *d*-glucose by yeast extract at 25° and at 10° was observed only when the yeast is dried at a temperature not exceeding 28° and then extracted at 25°. With higher temperatures the rate of mannose fermentation was invariably lower than that of glucose (Table 4). This is in conformity with earlier findings of Slator and of Jephcott & Robison. Slator (1908) reported that exposure of living yeast to heat diminishes its capacity to ferment mannose to a much greater extent than its capacity to ferment glucose. Jephcott & Robison (1934) (cf. also Neuberg & Ostendorf, 1930) discovered that mannose-6-phosphate, formed when brewer's yeast, dried at 37° for 24 hr., acts upon *d*-mannose at 38°, may account for almost the whole of the hexose monophosphate fraction and more than 80 % of the total ester P. With *d*-glucose 80 % of the phosphorylated products are represented by fructose diphosphate and only 20 % by the monophosphate fraction, the latter consisting mainly of a mixture of fructose-6-phosphate and glucose-6-phosphate together with minute amounts of mannose-6-phosphate. These results suggest that the conversion of the individual aldo-hexose-6-phosphate to the common intermediate fructo-

$$\text{Glucopyranose-6-phosphate} \underset{\text{Isomerase I}}{\rightleftharpoons} \text{Fructofuranose-6-phosphate} \underset{\text{Isomerase II}}{\rightleftharpoons} \text{Mannopyranose-6-phosphate}$$

furanose-6-phosphate is catalyzed by two specific hexose phosphate isomerases, isomerase II being more readily destroyed between 28 and 38° than isomerase I. More evidence, however, is necessary to prove the existence of the two isomerases.

Recently, Berger, Slein, Colowick & Cori (1946) and Kunitz & McDonald (1946) have shown that the hexokinase reaction proceeds with mannose at about half the rate with *d*-glucose. This may account for the longer time taken to reach the maximum rate in the fermentation by yeast juice at 25° of mannose as compared with glucose (cf. Fig. 1). The equality of the two maxima is best explained by the previous observation that at low phosphate concentration (0·040 M) the reaction

3-phosphoglyceraldehyde + phosphate + cozymase
⇌ 1:3-diphosphoglyceric acid + dihydrocozymase

controls the over-all rate of hexose fermentation by dried yeast (Gottschalk, 1946).

It would thus appear that the observed differences between the fermentation of *d*-mannose and *d*-glucose by yeast cells and by yeast preparations over the range of temperature tested (0–38°) may be due to: (1) the slower rate at which mannose penetrates the cell-membrane, and/or (2) the slower rate at which mannose reacts with hexokinase, and/or (3) the instability of mannose-6-phosphate isomerase at temperatures above 28°.

SUMMARY

1. In contrast to that of *d*-glucose, the temperature coefficient of *d*-mannose fermentation by yeast cells is dependent on substrate concentration; it increases considerably with decreasing mannose concentration. With yeast extract as fermenting agent no such difference between mannose and glucose is observed. It would thus appear that the cell membrane is responsible for this phenomenon.

2. The maximum rates of mannose and glucose fermentation at 25° by yeast extract are the same provided that during the preparation of the yeast extract the temperature does not exceed 28°; with higher temperatures mannose is fermented markedly slower than glucose. Taken with previous findings of Jephcott & Robison (1934) this result suggests the existence in yeast of two aldo-hexose phosphate isomerases, the mannose-6-phosphate isomerase being more readily destroyed at 28–38° than the glucose-6-phosphate isomerase.

3. Experiments at 10° afford evidence that both α- and β-*d*-mannopyranose are fermented without preliminary interconversion by mutarotation.

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The Effect of Diet on the Glutathione Content of the Liver

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It has been shown recently (Glynn, Himsworth & Neuberger, 1945) that white rats kept on a diet containing all the amino-acids occurring in proteins with the exception of methionine and cystine, develop after varying lengths of time massive necrosis of the liver. Although it is not quite certain that the dietary deficiency of sulphur-containing amino-acids was the sole cause of the liver lesions observed, the results suggested that the proneness of the rats to develop hepatic necrosis was specifically associated with a lack of cystine, or its potential precursor methionine, in the amino-acid mixture. It seemed likely that the absence of these amino-acids from the diet would produce a reduction in the relative concentration of the non-protein sulphur in the tissues. Thus Lee & Lewis (1934) found in rats, which had been reared on a diet relatively deficient in cystine, low sulphur values in protein-free extracts of liver, kidney and muscle; these authors also observed that the ratio of

nitrogen to sulphur in such extracts was very much higher than normal, indicating a relatively greater loss of sulphur than of nitrogen. It was noted that the composition of the proteins of these tissues was not altered. In dogs also, protein depletion is associated with an increase of the ratio of total nitrogen to total sulphur in the liver (Miller & Whipple, 1942). Moreover, the results obtained by French workers (Randoin & Fabre, 1927, 1931; Binet & Weller, 1934, 1935; Marenzi & Braier, 1934) with the not very specific iodometric method, indicated that a definite decrease of the glutathione content of many organs, especially of the liver and kidney, is produced by starvation or administration of a diet poor in cystine or methionine. It was therefore proposed to investigate in some detail the effect of the diet and particularly of changes in the amounts of cystine and methionine supplied, on the glutathione content of the tissues. It was thus hoped to establish an association between variations in the chemical composition of the liver and the onset of hepatic necrosis.

In the course of this work the wider aspects of the problem became apparent. It was pointed out by one of us (Neuberger, 1946) in a discussion on the metabolism of amino-acids, that the clinical picture produced by dietary deficiencies of the various essential amino-acids is on the whole remarkably uniform. This is to be expected in so far as a deficiency leads only to an interference with the synthesis of proteins. Since the amino-acid composition of any particular species of protein molecules is fixed, the utilization of amino-acids for protein production is interdependent. In certain cases the body may adjust itself to a deficiency in a particular amino-acid by synthesizing proteins relatively poor in the missing compound at the expense of other proteins. This has indeed been found for cystine deficiency which leads to a decrease in the cystine content of hair (Lightbody & Lewis, 1929). The morphological changes observed indicated that the proteins poor in sulphur were relatively increased by the dietary deficiency (Smuts, Mitchell & Hamilton, 1932). However, it may be expected that such an effect will be rare; it does not indeed, even in the case quoted, extend to the proteins of tissues like liver and muscle. With low-molecular metabolites which are produced from essential amino-acids such an interdependence does not exist and a deficiency may therefore lead to a decrease of the concentration of the substance in the tissues. Specific lesions may therefore be expected to be associated with chemical changes in body constituents other than proteins. Lack of sulphur-containing amino-acids in the diet appears to produce effects which are not only more severe than those observed in deficiencies of other amino-acids, but the symptoms also seem to be qualitatively of a different character. It is to be expected therefore, that this difference would be reflected in a change of the relative composition of the body, particularly with regard to the non-protein constituents of the tissues.

EXPERIMENTAL

Young black and white or Wistar albino rats usually weighing 60–90 g. were used.

Diet

Unless otherwise stated the animals were given a stock diet which supported good growth (about 2·7 g./day) and contained 18–20% protein, mainly derived from dried milk and wholemeal flour. The low-protein diet had the following composition: salt mixture (no. 2, *U.S. Pharmacopoeia*, 1942) 4·5%, fat (arachis oil and cod-liver oil) 12%, starch 83·5%. This diet was adequate except in protein of which the only source was 100 mg. of dried yeast which was added as a daily supplement to provide certain vitamins. The protein content of this yeast was 40–50% and the daily intake of protein (40–50 mg.) was therefore very small. Amino-acid supplements were mixed with the diet which was given in such amounts as to ensure complete consumption. Diets containing 10% of cystine or methionine consisted of a mixture of the amino-acid and the stock diet and were fed *ad libitum*. The 60% casein diet consisted of: casein 60%, salt mixture 5%, fat (arachis oil and cod-liver oil) 12%, starch 18%, dried yeast 5%, and was also fed *ad libitum*. Details of the diets in which the nitrogen was supplied by a mixture of pure amino-acids may be found in the papers of Neuberger & Webster (1946, 1947).

On the low protein diet the animals steadily lost weight at the rate of about 1·3 g./day and the diets containing 10% of cystine and methionine also caused a rapid loss of weight. The diet containing 60% casein supported growth to about the same extent as the stock diet.

Estimation of glutathione

The animals were killed some 2 hr. after removal of food, either by asphyxiation with coal gas or by a blow on the head. After puncturing the heart, the tissues were removed as rapidly as possible, washed free of blood and sliced into 2% salicylsulphonic acid contained in a weighed 'Potter homogenizer' tube. The whole was then kept in an ice-salt mixture until it was reweighed, thawed, homogenized and finally filtered. The extracts were then used for glutathione estimation for which three methods were applied.

Results were expressed in terms of mg./100 g. of tissue and in the case of the liver also in terms of mg./100 g. of body weight. This latter expression to some extent eliminates the variations due to change in liver weight.

(1) *Iodometric method.* This was an adaptation of the method of Fujita & Numata (1938) in which the interfering ascorbic acid was removed by addition of excess of a solution of 2:6-dichlorophenol-indophenol (0·025%, freshly prepared) and back titration of the excess with a standard ascorbic acid solution (0·0025% in 2% metaphosphoric acid, also freshly prepared) (cf. Kennaway, Kennaway & Warren, 1944). The glutathione was then titrated at 0–5° with a standard solution of potassium iodate (0·0005N in 2% salicylsulphonic acid) in the presence of potassium iodide (final concentration 0·5%). Recovery experiments suggested that this modified method is reliable.

(2) *Colorimetric method* (*Fujita & Numata*, 1939). The intensity of the absorption of the solution was measured in the Spekker absorptiometer using the Ilford Spectrum green filter. Contrary to the report of Herrmann & Moses (1945) we found that delay between the addition of nitroprusside and ammonia caused a marked reduction in the sensitivity of the method and the two additions were therefore made immediately before the reading was taken. With this precaution the method gave accurate and reproducible results with pure glutathione solutions above a concentration of 0·01 %.

(3) *Manometric method*. This method was devised by Woodward (1935) and depends on the coglyoxalase action of glutathione. Here it was found advisable to store the yeast glyoxalase preparation in evacuated ampoules at 0° in order to preserve the activity effectively.

Comparison of methods

Table 1 contains averages of a number of results obtained by the three methods. The agreement is quite good with normal livers, though the values obtained by the iodometric method are somewhat high, and in cases where the liver contained only small amounts of glutathione the difference became significant. Under such conditions the colorimetric method was also found unsuitable owing to the occurrence of an inflexion in the calibration curve of absorptiometer readings.

In the case of lung and intestine, results obtained by the manometric method were invariably lower than those obtained by the iodometric method.

The manometric method appears to be the most reliable because of its specificity. Aspartathione and *iso*glutathione are the only compounds so far discovered which are capable of simulating glutathione as coglyoxalase (Behrens, 1941) and these are not known to occur in the body. Unless otherwise stated, the results quoted in this paper were obtained by the manometric method. In no case was the determination of oxidized glutathione attempted. This omission was considered justified on the grounds that previous workers have found little or none of it in the tissues (Binet & Weller, 1935; Dohan & Woodward, 1939).

RESULTS

Glutathione content of tissues from normal animals. Values for the glutathione content of the liver of animals maintained on the stock diet are given in Table 2. The results show a wide variation as indicated by the standard deviation. There is, however, no significant difference between the two strains of rats or between the two sexes. Data for certain other tissues are given in Table 3. The

Table 1. *Comparison of the different methods used for the estimation of glutathione*

(The figures given are averages of a number of estimations. Each individual sample was assayed by three methods. Values are given in mg. of glutathione/100 g. of tissue.)

Tissue	No. of estimations	Method of estimation: Iodometric	Manometric	Nitroprusside
Liver {Normal	16	220	183	196
Liver {Low	7	76	54	—
Kidney	10	234	74	97
Lung	4	88	63	—
Intestine	6	129	81	—

The discrepancy between the manometric and iodometric methods was much more serious in the case of the kidney. It was thought that this may be due to the presence of an enzyme which rapidly liberates cysteine from glutathione. Such an enzyme has been found in rat kidney (Schroeder & Woodward, 1937), and we have observed that cysteine takes up more than one equivalent of iodine under the conditions of our titrations. In order to eliminate the effect of such an enzyme the treatment of the kidney was carried out as rapidly as possible, the tissue often being minced and frozen in salicylsulphonic acid within a minute of opening the abdomen. In spite of this, however, the discrepancy was not resolved. It is doubtful, therefore, whether the difference between the results obtained by the two methods is completely explained by the presence of cysteine but we have not investigated this matter further.

figures are averages of a number of determinations; for the kidney the manometric method was used, whilst for other tissues the iodometric method was employed. These results also showed some scatter but not to the same degree as those for the liver and the average figures agree with those reported recently in the literature by other workers (for references see Herrmann & Moses, 1945).

The effect of protein deficiency and fasting on the glutathione content of rat tissues. After being transferred to the low protein diet the animals lost weight and the glutathione content of the livers of both strains fell, as can be seen from Table 2. The initial fall was very rapid, the change after 1 day being quite marked. The values then decreased more gradually to reach, after about 10 days, a more or less steady level of some 2 mg./100 g. body weight. To test the significance of these changes the figures for both albino and black and white rats

Table 2. *Effect of fasting and dietary protein deficiency on the concentration of glutathione in the liver*

(Figures in the last column represent the mean ± the standard deviation.)

Strain of rats	No. of rats	Dietary regimen	Period on diet (days)	Liver weight (g./100 g. of body weight)	Liver glutathione (mg./100 g. of liver)	Liver glutathione (mg./100 g. of body weight)
Black and white	8	Stock	—	4·40	208	9·20 ± 4·32
	6	Fasting	24 hr.	4·30	231	10·20 ± 1·41
		,,	48 hr.			
					Combined	9·64 ± 3·40
	5	Low protein	1	4·12	151	5·96 ± 1·31
	5	,,	4–5	5·20	110	5·30 ± 1·49
	3	,,	14	4·00	44	1·41
	3	,,	16–17	4·80	45	2·14
	1	,,	23	3·06	67	2·05
Albino	9	Stock	—	5·42	174	9·16 ± 3·02
	2	Fasting	24 hr.	3·78	160	5·84
	1	,,	72 hr.	2·02	140	2·83
	2	Low protein	1	4·15	130	5·40
	4	,,	3–4	3·86	52	2·00
	2	,,	19	4·25	55	2·34

Table 3. *Effects of various diets on the glutathione contents of several tissues*

(The values are expressed in terms of mg. of glutathione/100 g. of tissue. Figures in parenthesis give the number of experiments from which each mean is calculated.)

Tissue	Stock diet: Mean	Stock diet: Range of values	Dietary regimen: Low protein diet given for 1 day	Low protein diet given for 3–5 days	Low protein diet given for 20 days	Dietary regimen: Low protein diet + supplement of cystine or methionine
Kidney	77 (10)	60–102	—	71 (5)	56 (2)	97 (6)
Muscle	50 (7)	40– 66	64 (2)	34 (2)	37 (1)	42 (4)
Lung	102 (7)	79–133	113 (5)	100 (5)	86 (2)	115 (3)
Intestine	121 (4)	114–132	119 (2)	115 (4)	—	140 (5)

were collected into four groups, those for rats on normal stock diet (20 animals, group A), and those for rats given low protein diet for 1 day (7 animals, group B), 3–5 days (9 animals, group C) and 14–23 days (10 animals, group D). The t values were calculated by the method of Fisher (1925) and found to be as follows for the differences between the mean values of group A and groups B, C and D respectively: 2·39, 4·03, 5·44. The difference after 1 day was therefore significant ($P < 0·05$) and the other differences highly significant ($P < 0·01$). The response of other tissues to a low protein diet has not been thoroughly investigated but the results presented in Table 3 suggest that any change which takes place in these tissues is of a less drastic and more gradual nature.

In contrast to the above result, fasting for 24 or 48 hr. had little or no effect on the glutathione content of the livers of black and white rats though there was considerable loss of body weight. The albino rats seemed to be somewhat more sensitive in this respect.

The effect of addition of cystine or methionine to a low protein diet on the glutathione content of rat livers. To determine whether cystine or methionine would affect the depletion of liver glutathione produced by the low protein diet, these amino-acids were administered with this diet in daily amounts of 30 and 37 mg./rat respectively. The results in Table 4 show that, when given from the beginning of the low protein period, these supplements were very effective and the levels of glutathione found in the liver appeared to be higher than the normal values. Similarly such supplements rapidly restored the liver glutathione content after depletion caused by varying periods of low protein diet. This latter effect was shown to be transitory, however. Thus, when the rats were fed on the low protein diet for 21 days and then given cystine or methionine for 2 days and returned to the low protein diet for a further 1 day before being killed, the liver glutathione had once more fallen to a very low level. The liver glutathione contents in four such experiments were (in terms of mg./100 g. body weight)

Table 4. *Effect of addition of methionine or cystine to a low protein diet on the glutathione content of the liver*

(Cystine was given in a dose of 30 mg./day and methionine in a dose of 37 mg./day. Each value represents one animal.)

Strain of rat	Period on diet (days): 1st period low protein diet	Period on diet (days): 2nd period low protein diet + supplement	Nature of supplement	Liver wt. (g./100 g. body wt.)	Liver glutathione (mg./100 g. liver)	Liver glutathione (mg./100 g. body wt.)
Black and white	0	6	Cystine	6·20	220	13·64
				5·80	260	15·10
	0	7	Methionine	5·84	345	20·15
				5·48	320	17·50
	4	1	Cystine	5·80	250	14·50
				5·60	312	17·50
	4	2	Cystine	5·40	280	15·12
				6·00	271	16·26
	4	7	Cystine	5·74	255	14·64
	4	1	Methionine	6·10	187	11·40
	4	2	Methionine	5·95	235	14·00
	4	7	Methionine	5·80	255	14·79
	21	0	—	3·06	66	2·02
	21	1	Cystine	3·75	282	10·60
	21	1	Methionine	4·65	312	14·51
Albino	19	0	—	4·25	45	1·92
				4·26	65	2·76
	14	1	Cystine	5·20	130	6·76
	14	2	Cystine	4·75	162	7·70
	14	5	Cystine	4·80	350	16·80
	14	1	Methionine	4·66	145	6·75
	14	2	Methionine	5·15	280	14·42
	14	5	Methionine	5·35	380	20·33

2·43 and 2·74 for cystine supplements and 3·03 and 4·34 for methionine supplements respectively. The results in the last column of Table 3 show that when either cystine or methionine was added to the low protein diet the glutathionine content of other tissues was well maintained.

Experiments with pure amino-acid diets. The specificity of the relation between the dietary intake of sulphur-containing amino-acids and the liver glutathione was established more firmly by experiments in which the nitrogen of the diet was supplied in the form of mixtures of pure amino-acids. The results of these experiments are shown in Table 5. The full diet supported good growth and maintained a normal concentration of glutathione in the liver. When both cystine and methionine were omitted for periods varying between 35 and 42 days, the glutathione content of the liver was reduced in four animals out of six to nearly the same level as that produced by prolonged protein deficiency. In two rats, however, the values found were very much higher than expected. With cystine alone the glutathione content of the liver remained within normal limits. In contrast, the omission of histidine, another essential amino-acid, from the mixture for 34 days had little effect on the glutathione content of the liver. The two low values, in terms of mg./100 g. body weight, are probably explained by the great reduction in liver weight relative to body weight observed in these animals.

Table 5. *Variation of the glutathione content of the livers of rats fed on diets in which the nitrogen was in the form of mixtures of pure amino-acids*

(The amino-acid mixture was either complete (A), or devoid of both cystine and methionine (B), cystine (C), methionine (D) or histidine (E).)

Rat strain	Diet	Liver glutathione (mg./100 g. of liver)	Liver glutathione (mg./100 g. of body weight)
Black and white	B	60	3·30
		63	2·90
		49	2·49
	C	200	7·13
	D	250	11·80
Albino	A	152	7·99
	B	125	5·35
		40	2·18
		115	6·18
	D	250	15·60
		315	17·20
	E	260	9·30
		150	6·14
		220	7·63
		160	5·61

The effect of diets rich in cystine or methionine on the glutathione content of rat livers. Diets containing 10% of cystine or methionine did not produce a spectacular increase in the glutathione content of the liver in spite of daily intakes of 500–1000 mg. of cystine and 250–500 mg. methionine respectively. The results in Table 6 suggest that an increase over the normal values takes place initially but eventually the glutathione concentration appears to be normal or even slightly reduced. Consumption of a diet containing 60% of casein for 5 days appeared to have little effect.

Table 6. *Effect of diets rich in cystine or methionine on the glutathione content of the livers of albino rats*

(The figures in the final column are based on the results obtained by the manometric method.)

Diet	Period on diet (days)	Liver glutathione (mg./100 g. liver) Iodometric method	Colorimetric method	Manometric method	(mg./100 g. body weight)
10% cystine	1	578	264	250	11·20
	1	600	—	250	15·00
	2	525	267	225	11·90
	3	334	—	230	12·11
	5	—	157	162	5·55
10% methionine	1	275	—	145	9·00
	2	392	400	370	17·30
	3	339	—	170	7·41
60% casein	5	—	216	212	9·02
	5	—	281	285	15·60

In the experiments in which excessive amounts of cystine were given the results obtained by the iodometric method were much higher than those indicated by either of the other two methods. The better agreement shown between the manometric and colorimetric methods suggests that glutathione is the only thiol compound present, and as cystine takes up very little iodine under the conditions of our experiments, the occurrence of the high reducing value is difficult to explain. This matter has not been investigated further.

DISCUSSION

Though glutathione has only been isolated from a few tissues, it is generally thought to occur in most cells, in varying concentrations. The function of this tripeptide is not definitely known, but its biological importance is probably associated with its SH group. Waelsch & Rittenberg (1941, 1942), using isotopic nitrogen, showed that glutathione is rapidly broken down and resynthesized in both intestine and liver; these authors calculated that half of the glutathione of the liver was replaced in 2–4 hr. Our findings, which indicate a very marked and quick response of the concentration of liver glutathione to dietary changes, are in keeping with their results. This lability causes a certain amount of difficulty in assessing the results of glutathione estimation. The wide variations encountered, even in normal animals, suggest that a single determination gives only a momentary glimpse of a rapidly changing situation.

The experiments with both low protein and pure amino-acid diets lead to the conclusion that the glutathione content of the liver depends entirely on the availability of cystine or methionine in the diet and not at all on the supply of other essential amino-acids. Thus administration of cystine to rats kept on a practically protein-free diet completely prevented the decrease of glutathione in the liver found on the basal diet alone, and similarly the loss caused by prolonged depletion was quickly made good by the addition of either cystine or methionine to this basal diet. The body can apparently supply the other two components of the tripeptide, viz. glutamic acid and glycine, fairly easily; this is also shown by the observation that administration of these non-essential amino-acids together with cystine causes an increase in the glutathione content of the liver which was not higher than that found with cystine alone.

Though variations in the glutathione content of the liver produced by changes of the diet are wide, they occur only within certain upper and lower limits. Thus a very marked increase in the intake of cystine or methionine has only a slight effect and the same also applies to an increase of proteins in the diet as a whole. Conversely, even in prolonged deficiency, glutathione never disappears from the liver, and indeed the average values found after a depletion of 19–23 days are not lower than those observed after a period of 14 days. It is likely that a steady state is established in which glutathione

is synthesized at a reduced rate in the liver from amino-acids obtained from the break-down of tissue proteins. Such a dynamic equilibrium between the sulphur-containing amino-acids present in proteins and the cystine in the glutathione of the liver may also explain the relatively high values of liver glutathione found in some animals on amino-acid diets deficient in methionine and cystine. In starvation, proteins are utilized to supply caloric requirements and are broken down more rapidly than in specific protein deficiency. It is likely therefore that in starvation larger quantities of cystine and methionine are available for glutathione synthesis and this probably explains our finding that fasting for a period of 24–48 hr. had little effect on the glutathione content of the livers of black and white rats. Binet & Weller (1935) subjected guinea-pigs to prolonged fasting (8 days) and found only a slight drop in the liver glutathione.

Our data, though not very extensive on this point, indicate that the glutathione content of other organs is not influenced by dietary variations to the same extent as that of the liver. Glutathione appears to share this lability with other constituents of the liver cell such as glycogen and the labile protein (Kosterlitz, 1944; Harrison & Long, 1945) and ribonucleic acid fraction (Davidson & Waymouth, 1944) of liver cytoplasm. It is doubtful, however, whether the decrease in the glutathione content of the liver is the only change in the relative composition of the body produced by a dietary deficiency of cystine and methionine. Thus the loss of non-protein sulphur found by Lee & Lewis (1934) in muscle and liver cannot be accounted for by glutathione alone and this applies also to the findings of Miller & Whipple (1942) in dogs. It is likely therefore that the concentration of some other sulphur-containing compound, present in many tissues in the non-protein fraction, is reduced in dietary deficiency.

The original purpose of this investigation was to find out whether the acute massive necrosis of the liver observed in rats reared on diets containing only traces of cystine and methionine is caused by a change in the composition of the liver with respect to sulphur. Dent (1947) has shown that the relative amounts of cystine and methionine in the proteins of the liver are not reduced, even in animals in which hepatic necrosis is observed. The results of this investigation show that a diet which may produce liver lesions also reduces considerably the glutathione content of the liver and probably also the concentration of other low-molecular sulphur-containing compounds. However, in the present experiments, hepatic necrosis was produced neither in the albino nor in the black and white rats, though the animals lost nearly half their original body weight during the period of deficiency. It is therefore impossible to state whether the onset of acute necrosis is associated with a further decrease or virtual disappearance of glutathione in the liver. It is doubtful, and in our opinion unlikely, that a decrease in the concentration of glutathione or any other sulphur-containing compound in the liver is by itself responsible for liver lesions. It is more likely that a toxic agent of an infectious nature, such as a virus, or a substance which arises in the normal metabolism of the animal, is involved. A deficient diet is likely to make the animal more susceptible to such a toxic agent and this effect may be associated with a reduction of the glutathione of the liver. The increased sensitivity to various toxic substances of animals kept on diets in which the intake of sulphur-containing amino-acid is the limiting factor has been demonstrated by several workers (Miller & Whipple, 1942; Heppel, Highman & Porterfield, 1946). The fact that the liver is the organ involved in these toxic effects may be connected with the marked lability of the liver glutathione in response to dietary changes.

SUMMARY

1. A diet low in protein produced in rats an almost instantaneous and marked reduction in the liver glutathione content which after about 14 days reached a constant level corresponding to 20–30 % of the normal value. This fall was prevented by the addition of cystine or methionine without a corresponding increase of the intake of other amino-acids. Administration of cystine or methionine also restored very quickly to normal low glutathione values produced by a deficiency. Other organs were not greatly affected.

2. An increase in the intake of cystine or methionine to a high level did not produce a marked effect on the glutathione content of the liver as measured by a colorimetric or manometric method, though a considerable increase was indicated with the iodometric method. Starvation appeared to affect the glutathione content of the liver to a lesser extent than protein deficiency.

3. Similar results were obtained with diets in which nitrogen was supplied in the form of pure amino-acids. It is concluded that the glutathione content of the liver depends solely on the intake of cystine or methionine. The bearing of these results on the dietary production of hepatic necrosis is discussed.

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The Folic Acid Group of Growth Factors

1. THE COMPLEXITY OF GROWTH FACTORS FOR *STREPTOCOCCUS LACTIS* R

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Considerable confusion has arisen with regard to the various preparations which have been reported as having activity for *Streptococcus lactis* R. In the main this has been due to a desire on the part of many workers to over-simplify the question by claiming that preparations obtained by different methods from a variety of sources contain identical substances. The complexity of the subject was obvious, however, as soon as it became possible to compare the activity of the liver fractions of Bohonos, Hutchings & Peterson (1941) and of Stokstad (1943), with a yeast-derived sample obtained by the last worker, and one isolated from fermentation residues by Bohonos, Hutchings, Slobodkin & Stokstad (1944). Comparative data for these preparations, and for other active materials whose preparation and properties have since been reported, are given in Table 1.

Recently Johnson (1946) published data for *Lactobacillus casei* and *Strep. lactis* R, claiming to show that folic acid, Vitamin B_c and the *L. casei* factor (both synthetic, i.e. pteroyl-glutamic acid and natural, i.e. the preparation from liver) are identical, a conclusion which does not appear to be completely justified in view of the data of Table 1. Since synergism has been observed between various fractions isolated from liver (Chattaway, Dolby & Happold, 1944), it was decided to examine the effect of the simultaneous addition of pairs of various growth factor preparations to culture media, using *Strep. lactis* R as test organism. This organism was chosen in preference to *L. casei* because, using a medium based on an acid-hydrolysate of casein as generally employed in these laboratories, it has not been found possible to stimulate good growth of *L. casei* by the sole addition of one of the 'folic acid group' of factors. Simultaneous addition of a pair of growth-factor preparations, separately apparently identical as tested by the growth response of *Strep. lactis* R, might indicate differences in composition, whether the individual growth-factor preparations were single or complex

Table 1. *Comparative data for the folic acid group of factors*

Name of preparation	Source	Activity for (Amounts for half max. growth μg./ml. medium) *L. casei*	Activity for (Amounts for half max. growth μg./ml. medium) *Strep. lactis* R	Ultra-violet spectrum	Other properties	Reference
Norite eluate factor *L. casei* factor	Liver	0·000055	0·00025	Not like xanthopterin	Contains pteridine nucleus but apparently not the same structure as xanthopterin	Bohonos *et al.* (1941)
Do.	Yeast	0·000055	0·00050	Not like xanthopterin	—	Stokstad (1943)
Do.	Fermentation residues	0·000061	0·0042	Not like xantopterin	—	Bohonos *et al.* (1944)
Pteroyl-glutamic acid synthetic *L. casei* factor	Synthetic	0·00007	0·0003	Not like xanthopterin	Claimed as sole requirement of *L. casei*; not substantiated by Chattaway *et al.* (1944, 1945)	Angier *et al.* (1945, 1946)
Folic acid	Spinach	0·000072	0·000055	Xanthopterin-like	Claimed by Mitchell *et al.* to be like but not identical with xanthopterin	Mitchell *et al.* (1941)
Vitamin B_c	Liver	0·00005	0·00025	—	Suggested as identical with 'norite eluate factor' and folic acid	Binkley *et al.* (1943)
Vitamin B_c conjugate	Yeast	0·01	0·12	—	—	Binkley *et al.* (1944)
Strep. lactis factor	—	0·8	0·000034	—	Gives unstable folic acid-like material when incubated with *Strep. lactis*. Foster *et al.* (1944) and Larsen & Stokes (1945)	Keresztesy *et al.* (1943)
L. casei factors	Liver	Certain of these factors alone show activity for *Strep. lactis* but none for *L. casei*. The addition of two or more produces a medium capable of supporting the growth and stimulating the acid production of *L. casei*				Chattaway *et al.* (1944, 1945)

In view of the observations of Mitchell & Snell (1941), Stokes (1944), and Kreuger & Peterson (1945) that thymine could replace folic acid and vitamin B_c in the growth of *Strep. lactis* R and the claim of Chattaway (1944) that orotic acid was active for *L. casei*, these compounds have also been tested for synergism with the other naturally occurring factors.

METHODS

Organism. The strain employed in these experiments, *Strep. lactis* R (that employed by Mitchell, Snell & Williams (1941) for the assay of folic acid preparations), was obtained from the National Collection of Type Cultures (No. 6459). The stock was maintained by weekly subculture on sucrose agar slopes fortified with liver extract. The inoculum for each 10 ml. tube of medium consisted of a 0·05 ml. drop of a sterile water suspension of a 24 hr. subculture also on liver-sucrose agar (approximately 10^6 organisms/ml. of suspension).

Medium. The medium used was essentially that of Snell, Guirard & Williams (1942) for the assay of folic acid. The amino-acid complement was supplied by the inclusion of an acid-hydrolysate of casein prepared by the method of Johnson & Mueller (1941), with the addition of cystine, tryptophan, leucine and isoleucine (cf. Dolby & Waters, 1944). The medium was prepared in double strength and all additions were made before the final volume was brought to 10 ml., thus ensuring identical conditions for each culture.

Growth-factor preparations used. The following samples of preparations having considerable growth activity for *Strep. lactis* were available:

(1) A sample of a 7% folic acid concentrate, isolated from spinach by Prof. R. J. Williams.

(2) The synthetic *L. casei* factor (pteroyl-glutamic acid) prepared in the Lederle laboratories (Angier, Boothe, Cosulich, Fahrenbach, Hutchings, Hultquist, Kuh, Mowat, Northey, Seager, Semb, Sickels, Smith, Stokstad, Subbarow & Waller, 1945).

(3) A sample of vitamin B_c isolated by Binkley, Bird, Bloom, Brown, Emmett, Hogan, O'Dell & Pfiffner (1943) and supplied by the Parke Davis laboratories.

(4) A liver fraction prepared by Chattaway, Dolby, Happold, McMillan & Waters (1945): the

alumina eluate fraction of a baryta soluble portion of a liver extract.

(5) An extract prepared from poppy-straw by Hoffmann La Roche Ltd. (Basle), and claimed to be equivalent to Williams's folic acid. These preparations were kept in aqueous solution containing 1 or 10μg. of solid/ml. at 0°, and added to the medium, after suitable dilution, before autoclaving. It was proved by control experiments in which the additions were made aseptically after sterilization that none of these preparations was adversely affected by steam sterilization in the presence of the medium. Orotic acid and thymine were similarly added before sterilization.

Measurement of the effects produced. Except on a few occasions growth *per se* was not measured, the acid produced by a culture being titrated with 0·05 N-NaOH to pH 7·4 instead. It has been shown (Luckey, Briggs & Elvehjem, 1944; Hall, 1947) that acid production is not strictly parallel to growth; therefore no claim can be made that the results quoted below indicate changes in growth. Qualitatively, however, with the preparations tested, parallelism existed between growth and acid production; no effect similar to that described by Dolby, Happold & Sandford (1944), in which growth could be entirely separated from acid production, was noticed. The rate of acid production decreased rapidly as the pH of the medium fell, and the glucose utilization in such cases was never optimal. Maintenance of pH at a constant level during long periods of incubation by the continuous addition of alkali was not practicable owing to the alterations in rate of acid production which are apparent during a long period of growth (cf. Hall, 1947). Therefore, as a compromise when longer periods of growth were studied, the pH value was adjusted to 7·4 by the aseptic addition of 0·05 N-NaOH every 24 hr. This enabled a progressive record of the acid production to be obtained; in such cases the total amount of available glucose was ultimately utilized. The medium was sterilized by autoclaving for 10 min. at 10 lb./sq.in. The tubes of medium after inoculation were incubated at 30°.

RESULTS

The growth-factor preparations tested separately as promoters of acid production

All the preparations were tested independently for activity in stimulating acid production in growing cultures of *Strep. lactis* R. A range of concentrations of from 1μg. to $1 \times 10^{-9}$$\mu$g./ml. of medium was examined, thus enabling differences unnoticed by Johnson (1946) to be observed. Fig. 1 shows a typical acid production/concentration curve, after 18 hr. incubation, for a culture containing pteroyl-glutamic acid. The portion indicated by the arrows, is almost linear when plotted directly and not semilogarithmically; this is the portion of most use for assay purposes, and covers the concentration range examined by Johnson.

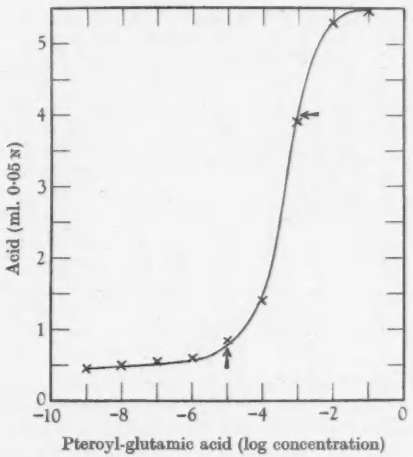

Fig. 1. The relationship between acid production and concentration of pteroyl-glutamic acid added to cultures of *Strep. lactis* R. Incubation 18 hr. at 30°. The portion between the arrows is almost linear if not plotted as a logarithm.

Table 2 shows a series of typical acid production figures for 24 hr. growth of cultures stimulated by a variety of factors. It will be seen that over the region of steepest slope, the acid production curves bear a marked resemblance to one another, differing only in respect of the concentration of the factors, which is affected only by the purity of the individual preparations. The figures for the extremes of concentration, however, do not show such a marked similarity. The upper limit of acid production towards which the figures asymptotically approached was not a constant figure, but varied between 3·90 and 6·15 ml. The concentration at which the point of inflexion occurred was not a fixed value either, the required concentration for half maximum acid production occurring at 3×10^{-6}, 1×10^{-4}, 3×10^{-6}, 6×10^{-1}, and 10μg./ml. of medium for pteroyl-glutamic acid, folic acid, vitamin B_c, the poppy-straw extract, and the liver fraction respectively.

There were differences at the lower extreme as well, folic acid always showing a step at the lowest concentrations. The activity in no case decreased gradually on the horizontal portion of the curve as with the synthetic material.

Table 2. *Comparison between the acid production induced in cultures of* Strep. lactis *R by the inclusion of various growth-factor preparations*

(Incubation period 24 hr. at 30°. Acid production is measured in terms of ml. of 0·05 N-NaOH needed to bring to pH 7·4, 10 ml. of culture. Blank for 10 ml. of medium without addenda = 0·30 ml. of 0·05 N-NaOH.)

Concentration of factor (Cols. 1–4, 1×10^{-x} μg./ml. medium) (Col. 5, 1×10^{-x} mg./ml. medium)	Acid production				
	Pteroyl-glutamic acid	Folic acid	Vitamin B_c	Poppy-straw extract	Liver fraction
(x)	ml. 0·05 N-NaOH				
0	—	3·90	—	6·15	—
1	5·90	3·40	—	1·30	3·90
2	5·95	3·10	3·95	0·50	3·60
3	5·85	2·20	3·65	0·25	2·80
4	5·80	1·90	3·00	0·20	2·10
5	5·25	1·50	1·90	—	1·40
6	1·45	1·30	0·90	—	1·20
7	0·70	1·20	0·65	—	0·90
8	0·40	1·20	0·65	—	—
9	0·20	1·10	—	—	—

The examination of the activity of pairs of growth-factor preparations

In the examination of pairs of growth-factor preparations, care was taken in the choice of concentrations in order that there might be no possibility of the composite activity producing an effect in excess of the direct sum merely because of the increased gradient of the middle portion of the curve. With this in view the concentrations were so chosen that the acid production figures associated with them lay on either the upper or lower horizontal portions of the curve.

1. *Pteroyl-glutamic acid and folic acid.* Table 3 shows the figures for the acid production of a series of cultures containing both folic acid and pteroyl-glutamic acid at a series of concentrations, together with corresponding figures for the separate preparations. The addition of a relatively high concentration of pteroyl-glutamic acid to high concentrations of folic acid had no significant effect (column 4) whereas when such an addition was made to lower concentrations of folic acid, synergism occurred (see 0·0001 and 0·00001 μg. levels of folic acid). The addition of much smaller concentrations of pteroyl-glutamic acid, however, had an effect which was at least additive over the whole range, and definitely synergistic in certain levels. The reciprocal effect which is indicated by the last two columns in Table 3, shows that the presence of large quantities of folic acid fixed the relative degree of acid production, and that alterations in the concentration of pteroyl-glutamic acid merely superimposed a slight variation on this. With lower concentrations of folic acid, however, the two acted synergistically, while the relative concentration of pteroyl-glutamic acid was the deciding factor. The effects produced at intermediate concentrations tended to be suspect on account of the considerations mentioned in the introductory paragraph to this section, but it is quite definite that synergism at least as far as acid producing capacity is concerned occurred between

Table 3. *The effect of folic acid and pteroyl-glutamic acid when added to cultures simultaneously*

(Acid production is measured in terms of ml. of 0·05 N-NaOH/10 ml. medium needed to bring to pH 7·4 after 18 hr. incubation at 30°. Blank titration for 10 ml. medium without addenda = 0·20 ml.)

Concentration of folic acid or pteroyl-glutamic acid (μg./ml.)	Acid production					
	Folic acid alone	Pteroyl-glutamic acid alone	Folic acid + pteroyl-glutamic acid		Pteroyl-glutamic acid + folic acid	
			0·00001 μg./ml.	0·0000001 μg./ml.	0·1 μg./ml.	0·0001 μg./ml.
0·1	2·56	—	3·05	2·95	—	—
0·01	2·28	2·55	2·88	3·15	3·95	2·74
0·001	1·92	2·45	2·59	2·06	—	—
0·0001	0·25	2·28	1·45	0·60	3·55	2·45
0·00001	0·00	0·76	0·94	0·25	—	—
0·000001	—	0·45	—	—	3·56	1·10
0·0000001	—	0·22	—	—	—	—
0·00000001	—	0·10	—	—	3·38	0·40

folic acid and pteroyl-glutamic acid at the lower concentrations.

2. *The relationship of the poppy-straw extract and the liver fraction to the other factors.* A pair of similar series were prepared containing the poppy-straw extract of Hoffmann La Roche Ltd. in addition to another preparation, either folic acid or pteroyl-glutamic acid. Tables 4 and 5 show the acid produced after 18 hr. incubation of cultures containing such additions. It can be seen from Table 4, that no synergism occurred between folic acid and the poppy-straw extract; in fact there was a slight inhibition when either was present in high concentrations. At lower concentrations summation occurred, but at no value was the combined activity greater than the sum of those of the separate factors. On the other hand there was slight inhibition when a high concentration of pteroyl-glutamic acid was added to the poppy-straw extract and less than the additive effect when a smaller concentration was given, but low concentrations of both preparations produced a synergistic effect. The composite activity in this case was nearly 50% higher than the sum of the two separate effects (see Table 5).

Table 4. *The effect of the simultaneous addition of poppy-straw extract and folic acid*

(Acid production is measured in terms of ml. of 0·05 N-NaOH/10 ml. medium needed to bring to pH 7·4 after an incubation period of 24 hr. at 30°. Blank titration for 10 ml. medium without addenda = 0·30 ml. of 0·05 N-NaOH.)

Additions	Acid production I	II	III	IV
10 μg. poppy-straw extract I	6·60	—	5·61	6·15
0·01 μg. poppy-straw extract II	—	0·50	—	0·95
0·1 μg. folic acid III	—	—	2·34	—
0·001 μg. folic acid IV	—	—	—	0·47

The liver fraction prepared by Chattaway *et al.* (1945) and the poppy-straw extract are both very impure. No claim was made that the liver material was homogeneous, but it has been shown that the majority of the folic acid activity as measured by the promotion of growth of *Strep. lactis* R is concentrated in this fraction of the original liver extract. Table 6 shows the effect of mixtures of this preparation with various other growth-factor preparations. It can be seen that this liver material showed a marked similarity in effect to pteroyl-glutamic acid giving synergism with the poppy-straw extract and with folic acid at low concentrations. There appeared, however, to be a factor present which caused a marked inhibition when large concentrations of the liver preparation were present. This had the effect of depressing the acid production induced by the simultaneous presence of 0·1 μg. of folic acid and 1 mg. of liver extract.

3. *Vitamin B_c.* Of the factors available from animal and plant sources, vitamin B_c, as a member of the former group, might be expected to show similar properties to pteroyl-glutamic acid and the

Table 5. *The effect of adding poppy-straw extract simultaneously with pteroyl-glutamic acid*

(Acid production is measured in terms of ml. of 0·05 N-NaOH/10 ml. medium needed to bring to pH 7·4 after an incubation period of 18 hr. at 30°. Blank titration for 10 ml. medium without addenda = 0·17 ml. of 0·05 N-NaOH.)

Additions	Acid production I	II	III	IV
10 μg. poppy-straw extract I	6·6	—	5·95	6·96
0·01 μg. poppy-straw extract II	—	0·45	—	1·54
0·001 μg. pteroyl-glutamic acid III	—	—	3·9	—
0·0000001 μg. pteroyl-glutamic acid IV	—	—	—	0·60

Table 6. *The simultaneous addition of liver extract* (*Chattaway* et al. 1945) *with other growth-factor preparations of the folic acid group*

(Acid production is measured in terms of ml. of 0·05 N-NaOH/10 ml. medium needed to bring to pH 7·4. Blank titration for 10 ml. of medium without addenda = 0·24 ml. of 0·05 N-NaOH. Incubation period 18 hr. at 30°.)

Additions	Acid production Alone	With 1 mg. liver extract	With 0·001 mg. liver extract
0·01 μg. pteroyl-glutamic acid	2·65	2·50	—
0·0000001 μg. pteroyl-glutamic acid	0·85	2·85	0·95
0·1 μg. folic acid	2·35	3·68	—
0·0001 μg. folic acid	0·32	3·39	0·98
10 μg. poppy-straw extract	6·46	5·85	—
0·01 μg. poppy-straw extract	0·55	2·96	1·30
1 mg. liver extract	2·80	—	—
0·001 mg. liver extract	0·55	—	—

liver fraction. Table 7 shows a set of typical figures for the simultaneous inclusion of vitamin B_c and both folic acid and pteroyl-glutamic acid. Synergism was not so marked with folic acid as might be expected, and inclusion of pteroyl-glutamic acid produced a result, the implications of which are discussed below.

Table 7. *The simultaneous addition of vitamin B_c and other growth-factor preparations to growing cultures of* Strep. lactis *R*

(Acid production is measured in terms of ml. of 0·05 N-NaOH/10 ml. medium needed to bring to pH 7·4 after 18 hr. incubation at 30°. Blank titration for 10 ml. medium without addenda = 0·05 N-NaOH.)

Additions of vitamin B_c (μg.)	Acid production						
		Folic acid (μg.)			Pteroyl-glutamic acid (μg.)		
	Alone	0·01	0·0001	0·000001	0·0001	0·000001	0·00000001
0	—	1·58	1·05	0·75	1·40	0·75	0·55
0·01	2·08	1·35	1·25	1·30	—	—	—
0·0001	1·70	1·56	1·60	1·55	1·20	1·15	0·75
0·000001	0·60	1·75	1·10	0·74	1·20	0·95	0·75
0·00000001	0·45	1·24	0·75	0·75	1·15	0·90	0·75
0·0000000001	0·46	1·30	0·75	0·72	—	—	—

Examination of the effect of thymine and orotic acid on acid production

Both thymine and orotic acid were examined for independent activity as promoters of acid production by *Strep. lactis* R; Table 8 gives typical titration figures. As claimed by Stokes (1944) and others, thymine had considerable activity as a factor inducing acid production by *Strep. lactis* R, and a fact noticed by Stokes (1944), but apparently ignored in his discussion of results, is particularly apparent from these figures, i.e. there was a lag phase during which comparatively little growth or acid production occurred, followed by a period of intense activity during which the initial drop below the comparable figures for folic acid was completely obliterated. Orotic acid, on the other hand, did not show the same degree of activity, and the amounts of acid produced by cultures containing 10μg./ml. were not significantly different from those produced by cultures, not containing addenda.

Table 8. *The effect of the addition of orotic acid and thymine to cultures of* Strep. lactis *R*

(Acid production is measured in terms of ml. of 0·05 N-NaOH/10 ml. medium needed to bring to pH 7·4 after incubation for periods indicated at 30°.)

Additions (μg./ml.)		Acid production		
		18 (hr.)	40 (hr.)	64 (hr.)
Orotic acid	10	0·41	1·15	1·55
	1	0·33	0·35	0·70
	0·1	0·27	0·29	0·50
Thymine	10	1·20	8·80	11·10
	1	0·90	6·90	8·10
	0·1	0·60	2·85	3·45
Nil		0·20	0·45	1·05

In the presence of other preparations, however, the results were very different. Table 9 shows the synergistic effects which were observed with orotic acid or thymine with folic acid, and the diminished if not altogether absent effect with pteroyl-glutamic acid. Orotic acid showed synergism with the higher level of folic acid tested, but appeared to be inactive, if not inhibitory, with the lower concentration. Thymine showed slight synergism at high levels, with the high concentration of folic acid, but not so pronouncedly at lower levels. With the highest concentration of pteroyl-glutamic acid there was no effect with orotic acid and inhibition

Table 9. *The synergistic effects produced by the simultaneous addition of folic acid or pteroyl-glutamic acid to media containing either thymine or orotic acid*

(Acid production is measured in terms of ml. of 0·05 N-NaOH/10 ml. medium needed to bring to pH 7·4 after 18 hr. incubation at 30°. 10 ml. medium in each culture.)

Additions of orotic acid or thymine (μg.)		Acid production					
			'Folic acid' (μg.)		Pteroyl-glutamic acid (μg.)		
		Alone	0·1	0·00001	0·0001	0·000001	0·00000001
Orotic acid	0	0·00	2·90	1·05	2·65	0·50	0·35
	10	0·05	4·25	0·98	2·60	0·85	0·46
	1	0·00	4·06	0·68	2·50	0·55	0·38
	0·1	0·00	4·04	0·60	2·45	0·55	0·30
Thymine	10	1·10	4·50	1·36	1·99	1·75	1·45
	1	0·85	4·00	0·96	2·45	1·40	0·55
	0·1	0·35	3·00	0·85	2·45	0·90	0·50

at high levels of thymine; over the rest of the range the effects were either additive in the case of thymine, or very slightly synergistic in the case of orotic acid.

DISCUSSION

The use of different preparations of the 'folic acid group' as standards in assay procedures has led to considerable confusion, due to differences which have been apparent in the results of analyses carried out by these methods. This has caused many workers to doubt whether all the various preparations which have growth-promoting activity for the lactic acid bacteria contain identical growth-promoting factors. In an attempt to determine whether assay procedures based on this variety of standards were comparable, Johnson (1946) showed that over the ranges usually employed in assays, certain of these preparations give identical growth/concentration curves. The fact that at concentrations outside this range, the growth/concentration, or acid production/concentration curves are not identical, indicates the possibility that these observations, taken over a narrow range on the steepest portion of the curve, are not necessarily a true picture of the relationship between these various preparations. There is, however, much stronger evidence in favour of the non-identity of the active materials in certain of these preparations in the synergism which occurs between pairs of them.

Since synergism occurs between folic acid and pteroyl-glutamic acid, it must be concluded that they are dissimilar. If it is conceded that folic acid is itself a mixture of factors it may still be held that one of these components is identical with pteroyl-glutamic acid. This is, however, unlikely since concentration considerations preclude there being any chance of a summation of effects between two portions of pteroyl-glutamic acid, one added in the folic acid and one already present, and their subsequent joint synergism with the remaining components of the folic acid preparation. The two impure preparations, from poppy-straw and from liver, apparently range themselves one on either side as far as their major constituents are concerned. Hence synergism is observed between pteroyl-glutamic acid and the poppy-straw extract, but not with the liver extract, and *vice versa* with folic acid. This division into two groups which can be classified with respect to the source of the preparation, i.e. one from liver, the other from plant sources, must also accommodate vitamin B_c which is obtained from liver. The effect of large concentrations of vitamin B_c with folic acid is to produce a marked inhibition, but as the concentration of vitamin B_c is decreased the composite effect increases rapidly, until at a concentration of 0·000001 μg./ml. of vitamin B_c an additive effect occurs, and at a concentration 100 times lower true synergism may be observed. Below this again the liver factor appears to be unable to stimulate the folic acid, and an average figure is produced. At lower concentrations of folic acid the peak occurs at a higher concentration of vitamin B_c, but does not reach a value high enough to constitute synergism. It appears, therefore, as if there may be some slight chemical difference between vitamin B_c and pteroyl-glutamic acid, which confers on the former the power of inhibiting folic acid to a certain extent while still maintaining its apparent identity with pteroyl-glutamic acid.

There is a significant difference between the absorption spectrum of folic acid and of pteroyl-glutamic acid, and hence it may be considered as likely that the above evidence is a true representation of the relationships between the various factors.

Stokes (1944) suggested that folic acid is not itself replaced by thymine, but that a thymine-like compound is the product of the reaction which utilizes folic acid. He also stated, however, that the activity observed after 40 hr. incubation with thymine was equivalent to that observed after 18 hr. when folic acid was used. This has been corroborated here, but by increasing the period of incubation it has been shown that the thymine-containing culture ultimately produced as much acid as the one containing folic acid, hence indicating that there is merely a lag in growth and acid production. It thus seems likely that the thymine may actually be a precursor of folic acid or that thymine is participating in some alternative metabolic path, since synergism is observed with folic acid, and there is no reason for synergism to occur between an enzyme and the product of its reaction. This is in agreement with the findings and conclusions of Hutchings, Falco & Sherwood (1945) who examined the effect of substituted uracils on the growth of *L. casei* and showed that there was a possibility that growth in the presence of folic acid does not entail the intermediary synthesis of thymine.

In view of the evidence advanced above, it may be better to consider the various members of the folic acid group of factors as independent entities bearing possibly close chemical relationship to one another, but having distinct physiological activities.

SUMMARY

1. A variety of preparations of the folic acid group of factors have been tested both separately and in binary mixtures for their effect on acid production by *Streptococcus lactis* R.

2. Evidence has been obtained which tends to show that folic acid (Mitchell, Snell & Williams, 1941) is not identical with pteroyl-glutamic acid (Angier *et al.* 1945, 1946) but that vitamin B_c (Binkley *et al.* 1943) may possibly be more similar to the latter than the former.

3. From a consideration of the effects of two impure preparations, one from liver and the other from poppy-straw, there appears to be a connexion between physiological properties and source, since the five preparations can be divided into two classes according as their sources are liver or plant tissue.

4. Synergism has been observed between folic acid and both thymine and orotic acid.

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The Folic Acid Group of Growth Factors

2. THE EFFECT OF FOLIC ACID ON ACID PRODUCTION BY GROWING CULTURES OF *STREPTOCOCCUS LACTIS* R

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It has been stated by Williams (1944) that folic acid, the growth factor preparation for *Streptococcus lactis* R and some other lactic acid producing bacteria, isolated by him from spinach leaves, is not a single entity but may consist of a group of compounds of similar chemical composition, but differing sufficiently to possess distinct physiological properties. Since it has also been suggested (Hall, 1947*a*) that none of the components of this complex is identical with preparations of similar activity isolated from liver, it appeared to be of interest to consider folic acid separately to deter-

mine whether it were unitary in nature, or if complex to examine any differences in activity between the various components.

METHODS

The medium, organism and inocula used in all the experiments reported below were as described previously (Hall, 1947*a*). Two preparations were employed: a 7·7% concentrate of folic acid isolated from spinach and kindly supplied by Prof. R. J. Williams and the synthetic material pteroylglutamic acid, claimed as identical with natural liver *L. casei* factor, prepared by the Lederle laboratories (cf. Angier, Boothe, Cosulich, Fahrenbach, Hutchings, Hultquist, Kuh, Mowat, Northey, Seager, Semb, Sickels, Smith, Stokstad, Subbarow & Waller, 1945, 1946). Both preparations were kept in stock aqueous solution containing 10 and 1 μg./ml. respectively, and after adequate dilution, added before autoclaving.

In most of the experiments it was desirable to continue incubation for considerable periods of time in order that stationary conditions of acid production should be reached. It has been stated (Elvehjem & Tepley, 1945) that growth of *Strep. lactis* ceases if the pH value is allowed to drop below 5·2, and to obviate this it was necessary to add alkali to the cultures in order to maintain the pH at 7·4. In view of the different rates of acid production which were encountered during a complete incubation, a constant dropwise addition was impossible; as a compromise alkali was added approximately every 24 hr. to bring the pH back to 7·4, using bromothymol blue as internal indicator. Sterile 0·05 N-NaOH was added aseptically, thus permitting progressive titration figures for the acid produced in any culture to be determined. Alkali as dilute as this was required in order that an accurate titration figure might be obtained. Although this entailed a threefold increase in volume before the end of the incubation, such a dilution was found to be immaterial since a series to which 40% (w/v) NaOH was added did not show any significant discrepancies in rate of acid production, nor in the ultimate value for the titration. Initially the pH of the medium which dropped to about 6·8 during the period of sterilization, was returned to 7·4 by the aseptic addition of alkali before inoculation; later this was obviated by arranging the reaction at 7·8, when the desired drop occurred during autoclaving without any change in activity of the medium.

The figures given for growth of the organism were obtained by a determination of the viable count by the dilution technique, inoculation of dilutions being made into 'Lab-Lemco' broth fortified by the addition of an acid hydrolysate of yeast. Strictly, this technique is not applicable to streptococci, because of morphological considerations. It was, however, shown that there was no change in average chain length between the beginning and end of a 14 days' incubation period. Hence it can be assumed that if the figures relating to growth are incorrect, they are so by a constant factor, and although absolute growth figures may not have much meaning, the changes in rate of growth will be adequately recorded.

Glucose utilization was estimated by determination of the residual glucose in a sample of the cell-free Seitz filtered medium. For this experiment 1 l. of medium was prepared, and samples withdrawn at intervals. Growth in these samples was immediately stopped by the addition of 0·1 ml. of 0·1% (w/v) solution of 'merthiolate' (sodium ethylmercurithiosalicylate). After removal of the cells by Seitz filtration, glucose determinations were made by Benedict's method.

RESULTS

To determine the mode of action of the folic acid complex, acid production/time curves were obtained for growing cultures containing a wide range of concentrations of the factor. Fig. 1 shows the

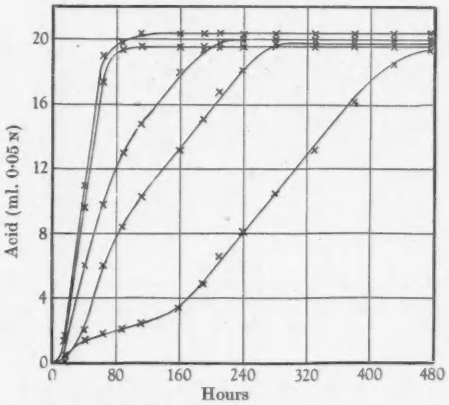

Fig. 1. Acid production/time curves for the growth of cultures of *Strep. lactis* R containing various concentrations of folic acid. Curves in order from the left refer to cultures containing respectively 1, 0·1, 0·01, 0·001 and 0 μg. of folic acid/ml. of medium.

type of result obtained. Given adequate incubation the ultimate acid production was independent of the initial concentration of folic acid but was equivalent to a total conversion of the glucose in the medium to lactic acid. The rates at which the acid was produced, however, initially varying in accordance with the concentration, changed after 60–90 hr. and if the glucose supply was not completely utilized, became equal to the rate at which acid was produced after a lapse of 7 days in cultures without added folic acid.

This apparent ability to dispense with added folic acid after 7 days' incubation was a consistent phenomenon. The rate of growth over the initial period in blank culture was dependent on the batch of medium used, possibly being conditioned by the purity of the casein acid-hydrolysate used. As can be seen from Table 1, however, the change to an increased rate of acid production occurred at about 160 hr. whatever the initial rate. The angles between the two portions of the curves are also independent of the initial rate of acid production, being in all three cases quoted in Table 1 equivalent to a change in rate of approximately 0·045 ml. 0·05 N-acid/10 ml. medium/hr.

Table 1. *Acid production by cultures of* Strep. lactis *R without added folic acid or pteroyl-glutamic acid*

(Acid production measured in ml. of 0·05 N-NaOH required to bring to pH 7·4 (bromothymol blue as internal indicator).)

Time (hr.)	Acid production: Culture 1 (ml.)	Culture 2 (ml.)	Culture 3 (ml.)
24	0·55	0·10	0·15
48	1·50	1·50	1·85
72	1·95	2·55	3·25
96	2·40	3·60	4·35
120	2·85	4·75	5·40
144	3·15	5·70	6·70
168	3·80	6·95	8·85
192	5·25	8·95	11·15
216	6·60	11·10	13·75
240	8·10	13·30	15·70
264	9·75	15·50	18·00
288	11·15	17·35	19·00
312	12·55	18·40	19·00

A series of curves, similar to those of Fig. 1, obtained by the addition of pteroyl-glutamic acid (Angier *et al.* 1945, 1946) is shown in Fig. 2. The

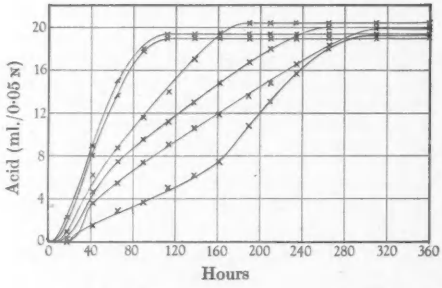

Fig. 2. Acid production/time curves for the growth of cultures of *Strep. lactis* R containing various concentrations of pteroyl-glutamic acid. Curves in order from the left refer to cultures containing respectively 0·001, 0·0001, 0·00001, 0·000001, 0·0000001 and 0 μg. of pteroyl-glutamic acid/ml. of medium.

same independence of ultimate acid production on the initial concentration of the factor can be observed, but there is not the same parallelism between the curves for the lower concentrations and that for the blank culture. In the former series there was a pronounced alteration in rate between 24 and 90 hr. incubation, after which the rate common to the lower concentrations set in. When the added factor is pteroyl-glutamic acid, however, there was a gradual falling-off in rate over the whole period of incubation.

In order to determine whether there was any correlation between growth rate, glucose utilization and acid production, a further series of experiments were undertaken in which the two former determinations were made in addition to the usual measurement of acid production. Medium (1 l.) was prepared and inoculated with about 5 million organisms in sterile water suspension. This volume of medium was aerated strongly (1 l./hr.) during the whole period of incubation, and samples were withdrawn at 24 hr. intervals. Table 2 gives the figures obtained for the various observations for a typical

Table 2. *Growth and metabolism of* Strep. lactis *R*

(Acid production measured in ml. of 0·05 N-NaOH/10 ml. culture required to bring to pH 7·4 (bromothymol blue used as internal indicator). Glucose content measured on samples removed after periods stated, and determined by Benedict's method. Viable count measured by serial dilution.)

Time of incubation (hr.)	Glucose remaining in culture medium (mg./ml.)	Acid production	Viable count (log value)
14	4·84	1·05	6·8
18	4·35	1·65	8·7
22	3·90	2·25	10·9
38	3·23	5·00	13·4
63	2·70	6·55	15·6
86	2·42	7·35	17·3

experiment. It will be noticed that between 24 and 90 hr. there was a falling-off in the rate of glucose utilization, which was mirrored by the change in viable count. The acid production figures also showed a change to the secondary constant rate, shown in Fig. 1. It is possible, therefore, that there is one mechanism responsible for carbohydrate metabolism during the early stages of growth, and another which functions later, and that acid production and growth although not strictly proportional to one another are definitely interconnected.

Attempts to separate the early and later activities of folic acid have been relatively successful, due to the discovery that quinoxaline has the power of inhibiting both growth and acid production of the organism. This compound, which may be considered as a xanthopterin nucleus with the nitrogen atoms of the pyrimidine ring replaced by carbons, appears to act as an antagonist to those growth factors containing a pterin system. Table 3 gives a series of acid production figures for cultures of *Strep. lactis* R containing quinoxaline and either folic acid or pteroyl-glutamic acid. It can be seen that both of these compounds, dissimilar in so far as they do not both show xanthopterin-like absorption spectra, were capable of reversing the inhibition caused by quinoxaline, due apparently to the pteridine, although non-xanthopteroid structure of pteroyl-glutamic acid. Large concentrations of quinoxaline were required for complete inhibition, but the

Table 3. *The effect of quinoxaline on acid produced by* Strep. lactis *R in the presence of folic acid or pteroyl-glutamic acid*

(Acid production is measured in ml. 0·05 of N-NaOH/10 ml. culture required to bring to pH 7·4. (Bromothymol blue used as internal indicator).)

Additions to basal medium			Incubation period (hr.)						
Quinoxaline	Folic acid	Pteroyl-glutamic acid	18	42	66	90	114	138	162
	μg./ml.		Acid production (ml.)						
—	—	—	—	1·2	2·25	3·40	4·60	5·50	6·40
1000	—	—	—	1·15	1·90	2·50	3·05	4·95	6·80
100	—	—	—	1·30	2·55	4·00	5·10	6·00	7·20
10	—	—	—	1·50	2·80	4·10	5·45	6·05	8·10
—	0·1	—	0·7	3·55	5·15	6·95	9·65	11·05	12·45
—	0·001	—	—	1·85	3·25	4·40	5·60	6·80	8·65
—	0·00001	—	—	1·60	2·90	4·25	5·40	6·60	7·85
—	—	0·01	1·0	8·50	13·80	18·45	19·00	19·00	19·00
—	—	0·0001	0·85	3·05	13·65	18·80	19·05	19·05	19·05
—	—	0·000001	0·30	4·65	7·50	9·65	11·50	13·05	14·85
1000	0·1	—	0·50	3·65	5·85	7·70	8·95	9·85	10·55
1000	0·001	—	0·25	0·35	0·75	1·40	2·70	4·70	7·10
1000	0·0001	—	0·20	0·30	0·70	1·35	2·65	4·80	5·85
1000	—	0·01	1·20	8·50	13·60	18·45	19·10	19·10	19·10
1000	—	0·0001	0·60	5·80	9·75	13·25	14·90	16·30	17·05
1000	—	0·000001	0·20	2·10	4·05	5·25	6·25	7·05	7·45
100	0·1	—	0·65	3·75	5·50	6·90	9·90	11·10	12·35
100	0·001	—	0·20	1·50	2·70	3·70	4·65	5·85	8·05
100	0·0001	—	0·15	1·35	2·45	3·55	4·25	5·90	6·40
100	—	0·01	1·20	8·90	14·70	18·50	19·00	19·00	19·00
100	—	0·0001	0·55	6·75	12·25	16·70	17·70	18·00	18·05
100	—	0·000001	0·30	3·75	5·95	7·10	8·70	10·00	11·35

results obtained are striking. Outside certain limits of concentration, either the inhibitory or the reversal effects may be paramount, giving either no growth and acid production or unimpaired activity, but between these limits, quinoxaline merely had the effect of impeding growth and causing a corresponding lag in acid production. Fig. 3 shows a series of curves relating acid production to time of incubation for cultures containing concentrations of quinoxaline and folic acid in this intermediate range. It will be seen that the quinoxaline merely depressed the activity during the period when the first rate of acid production (cf. Fig. 1) was operative. After a period depending on the concentration of both factor and inhibitor, acid production proceeded at an enhanced rate, but this alteration never persisted beyond the period after which, in the absence of quinoxaline, the secondary rate would supervene. Thereafter the acid production proceeded at the normal secondary rate, equal to that at which acid was produced in cultures not containing addenda. The only case in which there was evidence of an alteration in the time of onset of the secondary rate was when the quinoxaline was added to a culture without folic or pteroyl-glutamic acid. Under these circumstances acid production at the new rate began earlier.

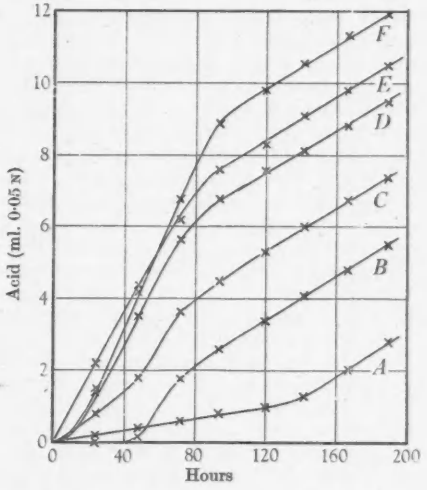

Fig. 3. The effect of the addition of quinoxaline on the acid production of cultures of *Strep. lactis* R containing various concentrations of folic acid. Additions in terms of mg. of quinoxaline and μg. of folic acid respectively as follows: *A*, Nil, Nil; *B*, 2·5, Nil; *C*, 2·5, 0·00001; *D*, Nil, 0·00001; *E*, 2·5, 0·001; *F*, Nil, 0·001.

DISCUSSION

There have been various indications of the possibility of folic acid being not a single substance but a group of closely related compounds with similar chemical but differing physiological activity. It has been difficult to reach any decision on this since there has been no standard simple substance with which folic acid could be compared. The preparation of synthetic pteroyl-glutamic acid, claimed to be identical with the liver *L. casei* factor by Angier *et al.* (1945, 1946), provided this criterion, since it may be assumed that the sole active substance present in this synthetic material is pteroyl-glutamic acid.

In view of this possibility of comparing folic acid with the pteroyl-glutamic acid, it is interesting to notice the differences between the acid production/time curves at various concentrations which are apparent from Figs. 1 and 2. The pteroyl-glutamic acid gives a series of curves, the slopes of which gradually fall away but which progress until the whole of the glucose supply is utilized, when a stationary state is reached. There is no abrupt change in rate such as occurs with folic acid after about 90 hr. incubation. One explanation of the change in rate of acid production observed after 90 hr. might be that folic acid is utilized stoichiometrically, and built *per se* into the structure of the developing cells with the concomitant expenditure of energy derived from the dismutation of the carbohydrate supply. This would of course mean that the amount of acid ultimately produced would be directly proportional to the initial concentration of folic acid, and would give rise to curves with plateaux at different levels. It would then have to be assumed that synthesis occurs thereafter at a constant rate causing the curves to rise at equal slopes until the whole of the carbohydrate source is utilized. This hypothetical onset of synthesis at the end of 90 hr., about 70 hr. before a similar effect is observable in the blank cultures, is the weak point in the hypothesis since there is apparently no reason why the presence of folic acid should induce an earlier onset of synthesis. This is borne out by the fact that the pteroyl-glutamic acid, although sufficiently different in physiological activity to act synergistically with folic acid (Hall, 1947*a*), is active in inducing similar degrees of acid production without a marked change of rate after 90 hr. Hence the possibility of folic acid being stoichiometrically utilized may be disregarded and other hypotheses considered.

Another possibility is that folic acid is in effect a mixture of two compounds, one of which is active during the earlier period of growth, the other later, and that during both these reactions the active material functions as part of an enzyme system and not as a substrate. In other words, although the rate of acid production is proportional to the concentration, the ultimate degree is entirely independent of this factor. Of these two components, it appears that the organism is only able to synthesize that which is active during the later stages of growth. The question of why the rates are identical during the second phase, no matter what the initial concentration, may be answered by assuming that the concentration of this component is sufficiently above the threshold value required for constant catalytic effect to obviate any proportionality.

Quinoxaline has been shown to have certain power as an inhibitor of the growth of *Strep. lactis* R. Large quantities are required but the results obtained with it are striking. The almost universal inhibition of the first of the two hypothetical reactions of folic acid, without any alteration in the secondary rate of acid production, may be significant. Quinoxaline was chosen as an inhibitor, on the assumption that the observations of both Woolley (1944) and Madinaveitia (1944) regarding the replacement of nitrogen atoms in adenine and riboflavine nuclei to give benzimidazole and a mepacrine-like structure, respectively, could be extended to those compounds containing xanthopterin-like structures. The activity of quinoxaline may not be indicative of the presence of a pterin structure, however, since histidine has a similar power of reversing the inhibition (Hall, 1947*b*). It seems possible, however, that the converse may apply. In other words any preparation which is active as a growth factor for *Strep. lactis* R but will not reverse the effect of the inhibitor may be assumed not to contain the structure to which the inhibitor is analogous. Hence, while for other reasons it is possible to say that the complete folic acid complex contains a pterin-like structure (Mitchell, 1944), it is also possible to say on the above basis that the second hypothetical component does not, and hence the former must be pterin-like in nature. The evidence may indicate the presence in folic acid of two components, the first of which, associated with the early period of active growth and acid production, may be pterin-like in nature, and hence allied to pteroyl-glutamic acid (although not identical, cf. Hall, 1947*a*), while the second, associated with the later stages of growth, does not contain a pterin-like structure. It is interesting to notice that the 'folic acid' produced by resting cells of *Strep. lactis* R from the substance obtained by Foster, Keresztesy & Stokes (1944) does not have the same properties as folic acid, being very unstable, and may be identical with the second component suggested above, which it has not been found possible to detect in the cell-free culture filtrate, a fact which may be due to its instability.

SUMMARY

1. Folic acid and pteroyl-glutamic acid have been examined for their ability to affect acid production by *Strep. lactis* R during a long period of incubation.

2. Evidence has been obtained pointing to the existence in folic acid of two factors with activity in the early and later phases of growth and acid-production.

3. Quinoxaline can act as an inhibitor of the growth of *Strep. lactis* R, and both folic acid and pteroyl-glutamic acid can reverse this inhibition.

4. Examination of acid production in the presence of intermediate concentrations of folic acid and quinoxaline is consistent with the presence of a pterin-like structure in the component responsible for early acid production, and not in the postulated component which is responsible for later activity.

The author wishes to thank Roche Products Ltd. for gifts of materials and for a grant in aid of this work, and Prof. F. C. Happold for helpful discussion and advice.

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The Folic Acid Group of Growth Factors

3. HISTIDINE AND ITS RELATIONSHIP TO 'FOLIC ACID'

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Since 'folic acid' was first described by Mitchell, Snell & Williams (1941) there have been repeated attempts to identify the activity of the natural preparations with compounds of known composition. On the basis of the absorption spectrum of the material, attention was drawn to the connexion between folic acid and xanthopterin (Mitchell, 1944). The work of Totter & Day (1943), Totter, Shukers, Kolson, Mims & Day (1944) and Totter, Mims & Day (1944), claiming the alleviation of nutritional cytopenia in monkeys, by the use of folic acid and to a certain extent xanthopterin, did much to establish this belief in the identity of the two compounds. Mitchell & Williams (1944), however, showed that xanthopterin could not replace folic acid in the growth of *Strep. lactis* R nor was folic acid as effective as xanthopterin in fish anaemia. It was also shown that of a large group of compounds with a high nitrogen content, only thymine was able to replace folic acid for *Strep. lactis*. This fact has been proved and examined by both Stokes (1944) for folic acid and Kreuger & Peterson (1945) for vitamin B_c, the former claiming that thymine does not directly replace folic acid, but that a thymine-like compound is the product of a reaction in which folic acid plays a part. On the other hand, in view of certain of his results, and those reported by the present author in a previous paper (Hall, 1947*a*) it appears more likely that if thymine is directly connected with folic acid, it may more readily be supposed to be a precursor than a product of folic acid activity. Chattaway (1944) examined a number of pyrimidine derivatives as growth factors for *L. casei*, and showed that orotic acid was active in this respect. It has also been shown (Hall, 1947*a*) that orotic acid, although not active alone for *Strep. lactis*, showed synergism with folic acid, as also did thymine. This tended to support the other possible conclusion that the active pyrimidine derivatives do not replace folic acid, but are in effect offering an alternative route for metabolism.

Many workers have shown that in addition to folic acid, both *L. casei* and *Strep. lactis* R require the presence of a selection of purines and pyrimidines (Snell & Peterson, 1940; Feeney & Strong, 1942; Snell & Mitchell, 1941), from which it would appear that heterocyclic nitrogen compounds play a considerable part in the nutritional requirements of these two organisms. Of other heterocyclic nitrogenous bases having biological activity, histidine is unique in that there are indications that it may act as a precursor for purines in mammalian metabolism (Degan, 1938). Although the evidence in this respect is rather confused, it appeared desirable to examine the action of histidine on the growth and acid production of *Strep. lactis* R to determine whether there is a similar connexion between it and purine or pyrimidine metabolism in the case of this organism.

METHODS

To obtain all the experimental results below, methods were employed as previously reported (Hall, 1947*a*, *b*). In all cases the organism employed was *Strep. lactis* R, although preliminary experiments had shown that similar effects could be observed on the addition of histidine to cultures of *L. casei*. The interpretation of the latter results was, however, more difficult, owing to the more complex growth requirements of *L. casei*. The medium and conditions of inoculation and incubation were identical in all respects to those employed during the investigation of the utilization of folic acid by *Strep. lactis* R and when the period of incubation was extended above the normal period of 18 or 24 hr. the method described by Hall (1947*b*) was used.

Histidine was added as an aqueous solution of *l*-(−)-histidine monohydrochloride, rendered neutral to bromothymol blue with NaOH before use. It was introduced into the medium either before sterilization or aseptically just before inoculation. All values given below for histidine content of the various cultures refer to the amount of histidine hydrochloride added. Owing to the fact that *Strep. lactis* R will not grow on a basal medium made from an amino-acid mixture without the addition of other uncharacterized factors besides folic acid, the histidine content of the casein-hydrolysate had to be taken into account. Analyses of the casein hydrolysate by an adaptation of the method of Kapeller-Adler (1934; cf. Chattaway, 1945) showed that within narrow limits, the histidine content of the basal medium amounted to 0·1 mg. of histidine/ml. Samples of *l*-(−)-histidine monohydrochloride were obtained from various sources, and some of them purified by recrystallization from 50% (w/v) aqueous ethanol. No differences in physiological activity were observed between crude and recrystallized specimens. Figures for the optical rotation of the purified material in a concentration of 2% (w/v) in N-HCl show a value for $[\alpha]_D^{20}$ of +8·8°.

Folic acid (a '7·7% concentrate' kindly supplied by Prof. R. J. Williams) was invariably added in aqueous solution prior to sterilization, which was carried out in steam at 15 lb./sq.in. for 10 min.

A sample of synthetic pteroyl-glutamic acid (Angier, Boothe, Cosulich, Fahrenbach, Hutchings, Hultquist, Kuh, Mowat, Northey, Seager, Semb, Sickels, Smith, Stokstad, Subbarow & Waller, 1945, 1946) was also used in certain experiments, being added to media in the required dilutions in aqueous solution before autoclaving.

RESULTS

The effect of histidine in the absence of folic acid

Various concentrations of *l*-histidine monohydrochloride were added to a series of tubes before autoclaving, and the results for titrations after 18, 40 and 64 hr. are shown in Fig. 1. The corresponding

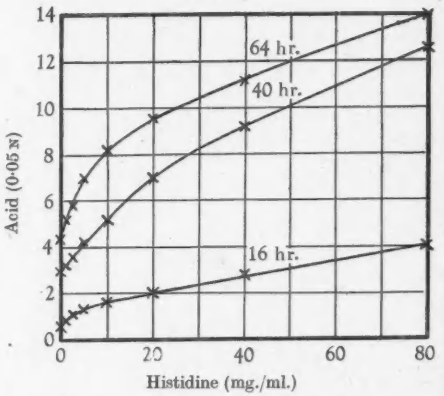

Fig. 1. The effect of histidine on the acid production of cultures of *Strep. lactis* R when grown in the absence of folic acid. Curves relating to different periods of incubation.

values for a culture containing no added histidine, but 0·01 μg. of folic acid/ml. are 4·55, 12·70 and 13·70 ml. 0·05 N-acid/10 ml. of medium, and for a culture with no addition 0·60, 2·95 and 4·40 ml. By a comparison of these figures with the curves in Fig. 1, it can be seen that when added in large quantities histidine gave the same degree of acid production as folic acid. The addition of 0·5 mg./ml. of histidine hydrochloride increased the acid production above that observed in the blank, but it was not until a concentration of 80 mg./ml. was reached that an increase in acid production comparable with that promoted by 0·01 μg. of folic acid was obtained. Histidine therefore, although potentially active as a substitute for folic acid, is 8×10^5 times less active at high concentrations. This ratio did not however remain constant over the whole range of concentrations. The rate of increase of acid production which was brought about by an increase in histidine concentration was such that when the concentrations of histidine and folic acid which separately induce identical degrees of acid

production are plotted against one another on a logarithmic scale (Fig. 2) the curves are linear in form. This indicates that the relationship between the molecular concentration of histidine (x) and of folic acid (y) (the molecular weight of folic acid being taken as 400 on the basis of the evidence of Mitchell & Williams, 1944) has the form of the equation $x = y^n$. The complex linear relationship between the log concentrations of the two additions indicates that there are two values for n, one of which is applicable at low concentrations, the other at high. These two values calculated from the slopes of the respective curves in Fig. 2, are 0·15 and 0·30, while the value for the comparison of histidine and pteroyl-glutamic acid which gives only one straight line over the considerable range of concentrations examined, is 0·60.

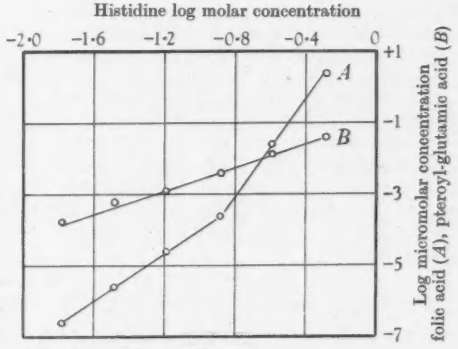

Fig. 2. The relationship between the log molar concentrations of histidine and two growth factors required to produce identical effects in the acid production of *Strep. lactis*.

The large concentrations of histidine, which were required to produce activity comparable to that of folic acid, may indicate that the activity is due to an impurity not chemically detectable but detectable by growth response. Experiments were carried out to determine whether such an impurity could be responsible for the activity.

Adequate acid production was obtained by the inclusion of 2·5 mg. of histidine hydrochloride/ml. of medium, and this concentration was employed to estimate the effect of steam sterilization on the acid production caused by such an addition. Pairs of tubes containing this concentration, added respectively before and after sterilization, were inoculated and incubated. Marked differences in turbidity and acid production were invariably observed. Typical figures for titrations carried out after 18 hr. on three separate pairs of tubes are given in Table 1. These figures indicate that when heated with the medium, histidine becomes active, being entirely inactive and in certain cases significantly inhibitory when unheated. As yet the component of the medium which may be considered responsible for the production of increased activity by reacting with the histidine during autoclaving has not been identified, since apparently none of the components of the medium has the power of activating histidine when autoclaved separately with this amino-acid. Adenine, guanine and uracil increased the activity slightly but not to the same extent as the complete medium.

Table 1. *The effect of steam sterilization on histidine activity*

(10 ml. quantities of medium were treated with 25 mg. of *l*-(−)-histidine monohydrochloride either before or after sterilization for 10 min. at 15 lb./sq.in. pressure. Acid production is measured in terms of ml. of 0·05 N-NaOH/10 ml. medium after 18 hr. incubation at 30°).

	Acid production		
Treatment	1	2	3
Heated	1·95	2·00	1·90
Unheated	0·45	0·40	0·50
Blank	0·80	0·60	0·80

Further evidence against the suggestion that the activity was due to an impurity is the fact that the *d*- and *l*-isomers were equally active. Acid production figures for pairs of cultures containing 5 mg./ml. of *d*- or *l*-histidine hydrochloride consistently fell around the following values after 18 hr. incubation: Blank 0·04 ml.; *d*-histidine 1·07 ml.; *l*-histidine 1·10 ml.

The effect of histidine and folic acid added simultaneously

The simultaneous addition of histidine and folic acid to the basal medium produces a variety of responses according to the concentrations employed.

Eight series of tubes were employed in a typical experiment containing folic acid in concentrations varying from 0·1 to 0·0001 μg./ml. and to each series histidine hydrochloride was added in concentrations varying from 0 to 5·0 mg./ml. The titration figures for the 8 series of tubes after 18 hr. are given in Table 2. Low concentrations of histidine had the power of inhibiting the growth and acid production of the organism when added simultaneously to certain sub-threshold concentrations of folic acid. When this threshold value of 0·001 μg./ml. of folic acid was exceeded, however, the inhibition was reversed and normal, or at higher concentrations enhanced, growth and acid production occurred. Even in the absence of folic acid histidine was inhibitory if administered in concentrations of less than 0·5 mg./ml. Since the basal medium contained

approximately 0·1 mg. of histidine/ml. the effect of complete absence of the amino-acid cannot be assessed, but minimal acid production occurred when the concentration of added histidine reached a level between 0·2 and 0·4 mg./ml. The inhibition which was observed at concentrations around this level was apparent over the whole of the period of incubation, and was not an increase in the lag phase followed by a period of enhanced activity, as occurs under other conditions (see below).

Table 2. *Acid production by* Strep. lactis *R in the presence of histidine and folic acid*

(Acid production was measured in terms of ml. of 0·05 N-NaOH/10 ml. medium to return to pH 7·4 after incubation for 18 hr. at 30°.)

	Additions of folic acid (μg./ml.)					
l-(−)-Histidine HCl (mg./ml.)	0	0·0001	0·001	0·005	0·01	0·1
	Acid production (ml.)					
0	0·70	0·80	1·05	1·55	2·65	4·70
0·1	0·50	0·60	1·10	1·60	2·70	5·10
0·2	0·45	0·55	1·15	1·65	2·75	5·35
0·4	0·60	0·75	1·30	1·80	2·95	5·60
0·5	0·75	0·95	1·40	1·85	3·05	5·75
1·0	0·90	1·15	1·65	2·20	3·45	6·16
2·5	1·15	1·30	1·70	2·35	3·90	7·30
5·0	1·30	1·60	2·75	3·05	5·30	8·90

When greater concentrations of histidine were added simultaneously with folic acid, inhibition occurred for the first 24 hr. but thereafter enhanced growth and acid production supervened, until ultimately full utilization of the glucose supply took place. Fig. 3 shows a series of curves relating histidine additions up to 80 mg./ml. to acid production, in cultures containing 0·001 μg. of folic acid/ml., after varying periods of incubation.

The inhibition observed in the presence of low concentrations of histidine was different from that produced by high concentrations, since the latter caused only an increase in the lag phase. Such a retardation of the onset of growth and acid production could be observed on the addition of a concentration of sodium chloride equivalent to that present in the cultures from the neutralization of the histidine hydrochloride. This increased lag was only apparent when folic acid was present in the medium (cf. Fig. 1).

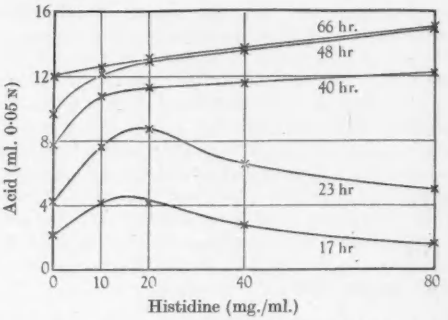

Fig. 3. The acid production induced by the addition of high concentration of histidine hydrochloride to cultures containing 0·001 μg. of folic acid/ml. of medium.

Although at the end of 18 hr. it appeared that histidine could raise the acid production above the level obtained with folic acid alone, it is apparent from the upper curves of Fig. 3 that the effect was transitory and was merely an increase in the initial rate of acid production, since the ultimate acid production was independent of the histidine concentration.

The reversal of quinoxaline inhibition

Tubes were prepared containing, in addition to the normal constituents of the basal medium, quinoxaline (2·5 mg./ml.) and histidine hydrochloride in concentrations from 2·5 to 10 mg./ml. The figures of the titration of the acid produced by such cultures after varying periods up to 143 hr. are given in Table 3. It will be seen that these concentrations of histidine were inadequate for complete reversal of the inhibition by quinoxaline, but a similar partial reversal was observed with sub-optimal concentrations of folic acid. The inclusion of greater concentrations of histidine with the

Table 3. *The reversal of quinoxaline inhibition by histidine*

(Acid production is measured in terms of ml. of 0·05 N-NaOH/10 ml. medium required to bring to pH 7·4 after incubation for the periods indicated at 30°.)

	Time of incubation (hr.)				
Additions (mg./ml.)	18	40	66	120	143
	Acid production (ml.)				
2·5 quinoxaline	—	0·15	1·75	3·35	4·15
2·5 quinoxaline + 10 histidine HCl	1·50	3·70	5·25	6·60	6·60
2·5 quinoxaline + 5 histidine HCl	1·15	2·65	3·80	5·35	5·35
2·5 quinoxaline + 2·5 histidine HCl	0·45	2·20	3·55	4·60	4·60
10 histidine HCl	1·60	5·20	8·20	11·15	13·35
5 histidine HCl	1·30	2·85	6·95	8·15	9·95
2·5 histidine HCl	1·15	3·60	5·85	7·35	8·85

object of completely reversing the inhibition would be inconclusive owing to the inhibition produced by the sodium chloride content (cf. Fig. 3). With concentrations up to 10 mg. histidine HCl/ml., however, there was a steady increase in reversal of the inhibition by quinoxaline.

DISCUSSION

It appears possible that folic acid can be replaced by histidine as a growth factor for *Strep. lactis* R. There is, however, some evidence to show that although histidine is the source of the activity, the amino-acid itself is not active—it is in fact inhibitory when added aseptically after sterilization of the medium, and even after heating with the medium, if added in small enough quantity. This may be due to the inadequacy of the active material formed under these conditions, to counteract the effect of the unchanged histidine. The evidence of Fig. 1 may indicate an equilibrium reaction between histidine and some component or components of the medium, the concentration of the final product being directly proportional to the concentration of added histidine.

The rate of increase of activity of histidine with concentration when compared with folic acid, as indicated by the slopes of the curves in Fig. 2, throws some light on the relationship between the factor produced from histidine and the folic and pteroyl-glutamic acids. The values for n of 0·15 and 0·30 indicate that below a certain level of folic acid a hundredfold increase in concentration of this preparation is required to produce an effect equal to a twofold increase in histidine concentration. Above this level the comparable factor is four. Evidence in favour of increased folic acid activity may be obtained by a comparison of histidine and pteroyl-glutamic acid, which when examined over the same range of histidine concentration as for folic acid (above) does not show any similar abrupt change, the value for n being constant over the whole range at 0·60, i.e. a hundredfold increase in pteroyl-glutamic acid concentration being equivalent to a sixteenfold increase in histidine. This may indicate that the synthetic pteroyl-glutamic acid maintains a constant activity ratio towards histidine, whereas the possibly complex (cf. Hall, 1946*a*) folic acid increases in relative activity when present in concentrations of more than 0·01μM. These observations answer the question of the origin of the activity associated with histidine, for if the activity were due to the presence of either folic acid or pteroyl-glutamic acid, the activity ratio would be unity. It is, therefore, reasonable to assume that neither of these two substances is responsible.

Since the suggestion of Stokes (1944) that thymine does not replace folic acid, but supplies preformed the product of the folic acid utilizing reaction, it was of interest to see whether the activity associated with histidine might agree with this suggestion. From the quinoxaline data it would appear that histidine is replacing folic acid as such or at least is supplying a product of the quinoxaline-inhibited step in the metabolism. It may equally well, however, be offering an alternative route for metabolism of glucose. If not identical, this route is similar, since the total amount of acid produced is independent of the presence of histidine, and lactic acid is always the sole product. The other observations, however, tend to indicate that a reaction product is formed by histidine with some component of the medium under the condition of sterilization.

Whatever the process by which this active substance is formed from histidine, it appears clear that it is playing an important role in the metabolism of glucose by *Strep. lactis* R. While it may bear no structural relationship to folic acid, it appears to maintain in operation a metabolic process not unlike that involving folic acid.

SUMMARY

1. The effect of histidine on the growth and acid production of *Strep. lactis* R, both alone and in the presence of folic acid has been examined.

2. Indications have been obtained of a reaction product between histidine and some component of the medium formed during autoclaving, which has activity similar to a member of the 'folic acid group' of growth-factors.

3. This material is neither folic acid nor pteroyl-glutamic acid.

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The Purification of Azoprotein Antibodies by the Dissociation of Specific Precipitates

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Antibodies can be separated from a part of the serum proteins by electrophoresis or by the fractional precipitation with ammonium sulphate. Purification by these or similar non-specific methods is limited by the fact that some of the serum proteins have properties almost identical with those of the antibodies. The separation of antibodies from such proteins is rendered possible, however, by the application of specific methods. These involve the precipitation of the antibody by the homologous antigen and the dissociation of the antibody complex into its components. The main difficulty consists in finding conditions, under which only the antibody is dissolved, while the antigen remains in the precipitate. This difficulty does not arise if cellular or bacterial antigens are used (Heidelberger & Kabat, 1938). Soluble antigens have been made insoluble by coupling them with stroma (Landsteiner & van der Scheer, 1936) or by adsorbing them on charcoal particles (Svensson, 1943). The antibody can be split off from these particles by dilute acetic acid. On the other hand, antibodies to the specific polysaccharides of pneumococci are extracted from the precipitate by lime water or baryta, while Ca or Ba compounds of the polysaccharide remain undissolved (Felton & Bailey, 1926; Heidelberger & Kabat, 1938).

Our own attempts to purify anti-azoproteins were based originally on the experience that azoproteins are precipitated by acids, while globulins (antibodies) remain dissolved (Haurowitz, 1936*a*). Accordingly we have attempted splitting of antibodies from the antigen-antibody precipitates by dilute acids.

EXPERIMENTAL

Preparation of antigens and immunization. Pseudoglobulin from beef or sheep serum was prepared as described previously (Haurowitz, Sarafyan & Schwerin, 1941). The protein (2 g.) was coupled with 2 mmol. of anthranilic, sulphanilic, arsanilic acid or *p*-aminobenzylamine (Haurowitz, 1936*b*, 1942). The azoproteins were purified by reprecipitation and dialysis and were mixed with 1% of solid NaCl. They were injected into rabbits subcutaneously as described in the papers mentioned. The immune sera were stored at −12° and thawed out shortly before the addition of the antigen.

Precipitation of anthranilic acid-azoglobulin by buffer solutions. A 5% solution of this azoprotein (0·2 ml.) was mixed with 2 ml. of the standard buffer solution of Teorell & Stenhagen (1938) and adjusted to the intended pH by the addition of the required amount of 0·1 N-HCl. The volume was adjusted to 2·5 ml. by the addition of water and the mixture kept at toom temperature for 24 hr. The tubes were then centrifuged and the amount of azoprotein in the supernatant solution determined colorimetrically. The amount of precipitated azoprotein was obtained as the difference between the total and the dissolved azoprotein. The results are shown by the following figures:

pH:	2·0	2·5	3·0	3·5	4·0	4·5	5·0
Precipitated azoprotein (mg.):	0	4·2	5·4	6·2	0	0	0

Precipitation of the antibodies. Different amounts of the immune sera, varying from 3 to 55 ml., were precipitated by the addition of solutions containing from 1 to 3% of the homologous antigen. The antigen solution was added either in a single dose or fractionally, in amounts of 0·05–3·0 mg., until no further distinct precipitate was formed (Haurowitz, 1942). Some of the sera (Table 1, no. 263) were precipitated in both ways in parallel experiments. The precipitates obtained by the fractional addition of the antigen were

combined, homogenized by stirring with a glass rod and then treated as the precipitates obtained by a single dose of the antigen. Only in the exp. no. 256 (Table 1) were the combined precipitates nos. 1, 2 and 4 on the one hand and nos. 5–8 on the other, collected in two separate tubes and analyzed separately. The mixture of antigen and immune serum was kept at 38° for 30 min. and frozen solid. Having been kept at −12° over night the precipitates were thawed, homogenized and freed from soluble substances by repeated washing with 1% NaCl solution.

Dissociation of the precipitates. Preliminary experiments with anthranilic acid-azoglobulin had shown that a dissociation of the precipitate is not brought about by solutions of the Teorell & Stenhagen (1938) buffer at a pH between 4 and 7 or by concentrated solutions of NaCl (Heidelberger & Kendall, 1936). On the other hand the precipitates were completely dissolved by aqueous solutions of baryta or of HCl. The dissolution in HCl could be prevented by addition of solid NaCl. Accordingly the following procedure was adopted for the purification of antibodies.

The ppt. was mixed with *c.* 2–3 vol. of 10% (w/v) NaCl (0·5–6·0 ml.). The mixture was homogenized by stirring and cooled to 0°. Then an equal vol. (0·5–6·0 ml.) of a chilled solution of the acid indicated in Table 1 was added, the mixture homogenized immediately and centrifuged. No low-temperature centrifuge being available, the solution was centrifuged only for 2–3 min. at room temperature. The supernatant solution was poured into a centrifuge tube containing the amount of 0·1 N or 1·0 N-NaOH required for neutralization, and was adjusted to a pH of 7 by the dropwise addition of mono- or disodium phosphate, respectively (bromothymol blue being used as external indicator). The acid-treated ppt. was stirred up with 0·5–3·0 ml. of a 5% solution of NaCl and centrifuged. The supernatant liquid was added to the neutralized first extract and brought again to a pH of 7 by sodium phosphate. The bulk of the antibodies was extracted by this method. A second extraction with acid furnished only traces of antibody.

The neutralized antibody solution was clear and colourless. Previously it had been assumed, therefore, that it was free of antigen (Haurowitz & Tekman, 1946). Later experiments proved, however, that it contained an antigen-antibody complex; this was removed by diluting the solution with water to a content of 1·5% NaCl, keeping it at 38° for 30 min. and then freezing solid. The frozen solutions were thawed, homogenized and centrifuged. The precipitated antigen-antibody complex was combined with the acid-insoluble remainder. The supernatant solution, containing the purified antibody, was used for the analyses and experiments described in Table 1 and in the following sections.

The acid-insoluble remainders of the precipitates were mixed with 3–5 ml. of a 1% saline solution, homogenized by stirring, neutralized with disodium phosphate, washed with NaCl solution and organic solvents, dried, weighed and analyzed for antigen-content as described previously (Haurowitz, 1942). The results are recorded in Table 1.

Analysis of antibody solutions. The amount of total protein in the antibody solution was determined by Kjeldahl analysis on 0·5–2·0 ml. of the solution or in its trichloroacetic acid ppt. A nitrogen content of 16% was assumed for the antibody protein. The content of active antibody (Table 1) was determined by the repeated addition of 0·5–1·0 mg. of the homologous antigen to 3–5 ml. of the purified antibody solution, until no further distinct ppt. was formed. Two to four precipitates were thus obtained. They were combined, homogenized, washed and weighed; then their content of antigen was determined colorimetrically. The difference between the wt. of the ppt. and that of its content of antigen was considered as active antibody. Its amount was recalculated for the whole volume of the solution and recorded in Table 1 as active antibody.

As an example of an analysis, 10 ml. of the serum no. 279/83, after treatment of the ppt. with N-HCl, gave 16·9 mg. of an insoluble remainder containing 7·1 mg. of the antigen and 9·8 (=16·9–7·1) mg. of antibody. Kjeldahl analysis of the antibody solution (15 ml.) gave for the total amount of dissolved protein 28·4 mg. or 74% of the total amount of antibody precipitated from the immune serum, which amounts to 9·8+28·4=38·2 mg. Five ml. of the antibody solution, upon the addition of the antigen, gave 7·1 mg. of a ppt. containing 1·3 mg. of the antigen and 5·8 mg. of antibody. This corresponds to 17·4 mg. or 61% of active antibody in the total amount of dissolved protein.

These figures are minimal values, because a small excess of antigen was present after the last addition of the antigen, so that traces of soluble antigen-antibody complexes might have been formed. The actual amount of active antibody, therefore, is slightly higher than indicated in Table 1.

Further purification of the antibody. 25 ml. of the combined antibody solutions no. 279/83*a* (Table 1), containing at least 24·4 mg. of active antibody/36 mg. of total protein, were precipitated by the addition of 30 mg. of the homologous antigen. The ppt. was dissociated by 1·5 ml. of a 10% solution of NaCl and 1·5 ml. of 0·1 N-HCl, as described above. The resulting solution contained at least 17·3 mg. (*c.* 90%) of active antibody/19·2 mg. of total protein. Similarly, an antibody solution prepared from another immune serum (no. 259) to anthranilic acid-azoglobulin, containing 25·6 mg. of total protein with 42% of active antibody, upon precipitation and dissociation of the precipitate, furnished 9·6 mg. of dissolved protein containing at least 7·7 mg. (80%) of active antibody.

Multiplicity of the antibodies. The antibody solution recovered from the immune serum no. 270 (Table 1) was exhausted at first by the addition of beef serum globulin, then by arsanilic acid-azo-ovalbumin and, finally by arsanilic acid-azoglobulin. The amount of added antigen and of the recovered precipitates is recorded in the Table. Since the first test antigen (beef serum globulin) was not coloured, its amount could not be determined analytically. An antigen/antibody ratio of 1/3 was accepted as fairly probable.

RESULTS

According to the experiments described in Table 1 a considerable purification of azoprotein antibodies is accomplished by dissociation of the precipitates with dilute acids containing 5% NaCl. In most of the experiments 0·1 N-HCl was used, but equally good results were obtained with 0·1 M-phosphoric acid. A certain minimal acidity is required for a successful dissociation. The optimal pH depends upon the isoelectric point of the azoprotein. While antibodies to the feebly acid anthranilic acid-azoglobulin and to *p*-aminobenzylamine-azoglobulin

Table 1. *Dissociation of precipitated azoprotein-antibody complexes by acids*

(Acids and buffer solutions were 0·1 M unless otherwise stated. Serum no. 279/83 is a mixture of the four sera nos. 279, 281, 282 and 283. The figures recorded for the dissolved protein and for the active antibody in the experiments nos. 256 and 262 are slightly too high, because the small amount of dissolved antigen-antibody complex was not removed from the antibody solution. The following abbreviations are used throughout the Table: BGl = beef serum globulin, SGl = sheep serum globulin; buffer = mixture of 0·1 M-phosphoric acid and 0·1 M-KH_2PO_4. The ratio phosphoric acid/phosphate was 3/1, 1/1 and 1/3 in the buffer solutions A, B and C respectively. Buffer D is 0·1 M-KH_2PO_4. The pH of the solutions was determined by a quinhydrone electrode.)

Immune serum			Added antigen	Dissociating medium*		Insoluble remainder		Dissolved protein	Active antibody (as % of dissolved protein)	
No.	Homologous antigen	Vol. (ml.)	(mg.)	Vol. (ml.)	Composition	Total wt. (mg.)	Wt. of antigen (mg.)	(mg.)		Special remarks
237	Anthranilic acid-SGl	21·0	2 × 10	1·5	H_3PO_4	30·7	12·5	24·5	40	Active serum
237	Anthranilic acid-SGl	21·0	2 × 10	1·5	H_3PO_4	21·7	8·3	18·4	43	Inactivated serum
256	Anthranilic acid-SGl	25·0	4 × 3	5·0	HCl	23·2	8·0	17·7	82	Fraction 1 to 4
			4 × 3	5·0	HCl	22·6	10·0	15·2	44	Fraction 5 to 8
262*a*	Aminobenzylamine-SGl	7·5	3·5	0·75	CH_3COOH	11·1	5·7	7·6	23	(*a*) 11 Dec. 1945; (*b*) 20 Sept. 1946. The ppt. of 262*b* was extracted first with buffer C, then with N-HCl
262*b*	Aminobenzylamine-SGl	9·0	2 × 3·5	0·5	Buffer C	—	—	3·2	25	
				0·5	N-HCl	5·9	3·3	2·7	37	
263	Anthranilic acid-SGl	4·5	3 × 2	0·5	HCl	8·0	3·1	7·8	49	Precipitated fractionally
263	Anthranilic acid-SGl	4·5	6	0·5	HCl	8·5	3·3	8·6	37	Precipitated in a single dose
270	Arsanilic acid-BGl	8·0	3 × 9	1·0	HCl	18·5	8·0	29·4	7	Precipitated by BGl
									20	Precipitated by arsanilic acid-ovalbumin
									30	Precipitated by arsanilic acid-BGl
276*a*	Sulphanilic acid-SGl	5·0	2·5	0·5	HCl	5·4	2·1	9·5	14	(*a*) 1 July 1946; (*b*) 29 July 1946. The ppt. of 276*b* was extracted first with buffer, then with HCl
276*a*	Sulphanilic acid SGl	5·0	2·5	0·5	N-HCl	5·8	1·5	8·5	46	
276*b*	Sulphanilic acid-SGl	6·5	4	0·5	Buffer C	—	—	3·95	0	
				0·5	N-HCl	6·9	0·7	3·7	57	
278	Arsanilic acid-SGl	9·3	4 × 1	0·75	0·1 N-HCl	7·5	3·0	12·5	54	
278	Arsanilic acid-SGl	9·3	4 × 1	0·75	0·3 N-HCl	8·7	3·0	15·6	49	
278	Arsanilic acid-SGl	9·3	4 × 1	0·75	1·0 N-HCl	7·8	3·0	16·8	51	
279/83*a*	Anthranilic acid-BGl	10·0	12	1·0	N-HCl	16·9	7·1	28·4	61	(*a*) 3 Sept; (*b*) 5 Sept.; (*c*) 15 Sept. 1946
279/83*a*	Anthranilic acid-BGl	10·0	12	1·0	HCl	15·4	7·1	26·2	64	
279/83*b*	Anthranilic acid-BGl	10·0	12	1·0	H_3PO_4	15·2	6·0	19·0	67	
279/83*b*	Anthranilic acid-BGl	10·0	12	1·0	Buffer A	17·2	6·0	18·0	64	Buffer pH = 1·3
279/83*b*	Anthranilic acid-BGl	10·0	12	1·0	Buffer B	17·3	5·5	16·2	69	Buffer pH = 1·8
279/83*c*	Anthranilic acid-BGl	10·0	12	1·0	Buffer C	15·8	10·0	15·0	27	Buffer pH = 2·3
279/83*c*	Anthranilic acid-BGl	10·0	12	1·0	Buffer D	26·0	9·0	4·6	57	Buffer pH = 3·7

* An equal vol. of 10% (w/v) NaCl is added to the acid solution given.

are split off from the specific precipitates by the buffer solution C (pH = 2·3), antibodies to the strongly acid sulphanilic acid-azoglobulin are not extracted from the precipitate by this buffer solution (Exp. nos. 262, 276 and 279/283), and require the addition of HCl.

About 50–80 % of the precipitated antibody is split off from the precipitate by acids; on the other hand nearly all of the antigen and a part of the antibody remain undissolved. This method was applied successfully to nineteen different immune sera against anthranilic acid, arsanilic acid, sulphanilic acid and *p*-aminobenzylamine-azoglobulins. For brevity only some of these experiments are shown in Table 1.

A negative result in regard to arsanilic acid-azoglobulin, reported in a preliminary communication (Haurowitz & Tekman, 1946), was due to the very low titre of antibodies in the serum employed, as was stated later.

Only a part of the protein split off from the precipitate by acids is active antibody, precipitable by the homologous antigen. It was assumed, at first, that the inactivity of a part of the extracted antibody is due to its denaturation by the acid used for its dissociation. Our experiments (nos. 276, 278, 279/283) indicate, however, that the yield of active antibody is not reduced, when 0·1 N-HCl is replaced by 1·0 N-HCl. This does not indicate a strong denaturing effect of the acid. Possibly a part of the dissociated antibodies is composed of 'low-grade' antibodies which are not precipitated in the absence of more reactive antibodies (Heidelberger & Kendall, 1935). The presence of non-precipitable proteins cannot be attributed to non-specific proteins of the immune serum, since these proteins had been removed by repeated washing of the specific precipitates with saline solution. But one has to consider the possibility that a part of the complement protein is adsorbed to the precipitate and is split off by the acid. Attempts to reduce the amount of non-precipitable protein by keeping the immune sera at 56° for 50 min. and thus destroying the complement, had no success (Exp. no. 237, Table 1).

The yield of active antibody does not depend on the consistency of the precipitate, for it is not altered markedly by keeping the precipitate at 38° instead of freezing it or by the addition of the antigen in a single dose instead of adding it fractionally (serum no. 263). Higher yields of antibody were obtained, however, from the first than from the last fractions of the precipitate (Exp. no. 256, Table 1).

The experiments carried out with the immune serum no. 270 (Table 1) and similar experiments with other immune sera demonstrate that the solution of the purified antibody does not contain one or other of the antibodies present, but a multiplicity of antibodies adapted to the different determinant groups of the homologous antigen.

The dissociation of the specific precipitate by the acid-salt mixture had been ascribed previously (Haurowitz & Tekman, 1946) to the insolubility of the antigen at low pH values. This explanation is not satisfactory because anthranilic acid-azoglobulin is soluble at pH values < 2, as shown in the experimental part. Moreover, a part of the antibody cannot be extracted from the precipitate, even if the treatment with acid is repeated. An explanation is furnished, however, by recent experiments of Haurowitz, Schwerin & Tunç (1946), according to which azoproteins give non-specific precipitates with normal serum globulins at pH values under 5·0. We assume, therefore, that the specific precipitate, in which both components, antigen and antibody, are linked to each other by means of specifically adapted groups, on the addition of acid, is converted into a non-specific precipitate, in which azoprotein anions are bound to antibody cations by salt-like linkages. While specific precipitates contain 70–90 % of antibody, non-specific precipitates contain less than 50 % of globulin (Haurowitz, Schwerin & Tunç, 1946). Accordingly, considerable amounts of antibody are released from the precipitate by the addition of acid.

SUMMARY

1. Specific precipitates containing 10–30 % of azoprotein and 70–90 % of antibody are, on the addition of acids, converted into non-specific azoprotein-globulin precipitates, containing less than 50 % of antibody. About 50–80 % of the antibody is thus released from the precipitate and passes into the acid solution. Hydrochloric acid or phosphoric acid, containing 5 % of sodium chloride, was used for the dissociation of the precipitates.

2. Antibodies to anthranilic, arsanilic and sulphanilic acids and *p*-aminobenzylamine-azoglobulins were purified by this method. About 40–70 % of the dissolved protein was active antibody, precipitable by the homologous antigen. A repetition of the same procedure furnished antibody preparations of a high degree of purity containing at least 90 % of precipitable antibody.

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The Fate of Nicotinamide Methochloride and the Effect of Liver Poisons on its Elimination Rate in the Rat*

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The object of this work was to investigate two obscure aspects of the metabolism of nicotinamide, namely the fate of nicotinamide methochloride in the body and the effect of liver poisons on its formation and elimination. An important factor determining the rate of methylation of nicotinamide is probably the methylating efficiency of the liver. There are indications that in certain liver diseases in humans the urinary elimination of nicotinamide methochloride is affected (Najjar, Hall & Deal, 1945; Ellinger, 1945). The effect of carbon tetrachloride poisoning on the elimination of ingested nicotinamide methochloride by the rat has been studied by Perlzweig, Huff & Rosen (1946), who found elimination to be increased during the first days of poisoning. Ellinger & Coulson (1944) have shown that the recovery of nicotinamide methochloride from human urine is about 15% after oral and about 60% after parenteral administration; the remainder is unaccounted for. These findings have been confirmed by Perlzweig & Huff (1945), who postulated partial destruction in the liver (Perlzweig, Huff & Rosen, 1946), while Najjar & Deal (1946) suggested that hydrolysis of nicotinamide methochloride took place in the liver to yield methyl groups and nicotinamide.

To investigate these factors, the following experiments were carried out. The possible destruction of nicotinamide methochloride in the liver was studied (*a*) with liver slices and (*b*) with perfused surviving livers; (*c*) faeces and (*d*) bile were tested for nicotinamide methochloride; (*e*) the effect of faecal bacteria on the compound was examined and (*f*) the effect of liver poisons on the elimination of nicotinamide methochloride and protein nitrogen was investigated.

* Some of the results presented in this paper were communicated to the Biochemical Society on 4 May and 6 December 1946 (*Biochem. J.* **40**, xxxi and **41** (in the press)).

METHODS

(*a*) *Liver slices.* Albino rats of 250–400 g. of both sexes from our own stock, kept since weaning on a mixed diet and with a known daily nicotinamide methochloride output, were stunned and killed by bleeding. The liver was removed immediately after death and kept in a covered glass bowl. Pieces of 300–500 mg. wet weight were cut off, weighed, sliced into slices of 0·2–0·4 mm. thickness and suspended in 10 ml. of modified Ringer solution (Krebs & Henseleit, 1932) in sufficient weighing bottles to form a duplicate series; the solutions contained in addition 0, 1, 3, or 10 μg. of nicotinamide methochloride per ml. Suspensions were aerated with a mixture of 5% CO_2 and 95% O_2 before stoppering and controls prepared of each concentration without the addition of liver. All bottles were agitated at 37°. Never more than 30 min. elapsed between the death of the animal and commencement of shaking. Pairs of bottles containing liver were opened after 2, 30, 60, 120 or 240 min. respectively; after 4 hr. the blanks were opened. The supernatant liquids were decanted into graduated centrifuge tubes, the residues washed twice with 0·5 ml. water which was added to the centrifuge tubes. 1·0 ml. of 20% (w/v) trichloroacetic acid was added. After heating to 75° for 30 min. and adjusting the volume to 12 ml., the precipitate was centrifuged off and nicotinamide methochloride in the liquid determined by the method of Coulson, Ellinger & Holden (1944). On two occasions liver slices were agitated in Krebs-Ringer solution at 37° for 2 hr. before 3 μg./ml. nicotinamide methochloride were added. Determinations of this compound were made 2, 30, 60, and 120 min. subsequently.

(*b*) *Liver perfusion.* Rats of the same type were killed as in (*a*); the peritoneal cavity was opened, a cannula inserted

into either the hepatic artery or portal vein and the liver perfused with Krebs-Ringer solution at 37° after the hepatic vein had been severed. The liver was then removed, placed on a funnel and perfused first with Krebs-Ringer solution alone, and 20 min. later with the same solution containing 1, 3 or 10 μg./ml. of nicotinamide methochloride. The same 200 ml. of perfusion liquid were used during the whole experiment. The solution was examined at intervals for its nicotinamide methochloride content. The whole procedure was carried out at 37°. The perfusion pressure was 90 cm. water when the liver was perfused through the hepatic artery and 40 cm. when the perfusion was done through the portal vein.

(*c*) *Urinary and faecal elimination.* Rats of both sexes were kept in metabolism cages for about 2 months. Urine was collected daily and its nicotinamide methochloride content was measured. In addition, 24 hr. specimens of faeces were collected every other morning and dried *in vacuo* over $CaCl_2$ to constant weight. 3 g. or less of the dried faeces were suspended in 50 ml. 0·02% (w/v) HCl and kept in a boiling water bath for 2 hr. After cooling to about 40°, 10 ml. of a freshly made 5% (w/v) taka-diastase emulsion in sodium acetate-acetic acid buffer (0·1 M) of pH 4·8 were added and the mixture kept at 45° for 12 hr. After cooling to room temperature, the supernatant liquid was siphoned off, and nicotinamide methochloride estimated in measured portions. After a preliminary observation period, the rats were given either 10 mg. of nicotinamide or 10 mg. nicotinamide methochloride orally or intraperitoneally. In order to ascertain whether the bluish fluorescence of the *iso*butanol solution of the tests on faeces and bile was due to its content of the nicotinamide methochloride degradation product F_2 (Ellinger & Coulson, 1943; Coulson & Ellinger, 1943), its reaction towards strong alkali, potassium ferricyanide and alkaline acetone (Najjar, Scott & Holt, 1943) was examined.

(*d*) *Biliary excretion.* Male rats (150–450 g.) from various sources were anaesthetized by intraperitoneal administration of 0·07 ml. of nembutal solution (1 grain/ml.)/100 g. body weight. In some cases the dose had to be increased and small amounts had to be given about every hour to keep the animals fully unconscious. The peritoneal cavity was opened by a cut about 1 cm. long in the lower hepatic region and the duodenum carefully taken out on a gauze moistened with warm saline. The entrance of the common bile duct which is generally found near the distal end of the duodenum was then located. Ligatures were placed a few mm. on each side of the entrance and a cannula was fixed so that its tip almost touched the entrance of the bile duct. The intestines were then returned as completely as possible into the peritoneal cavity and the animal covered with gauze moistened with warm saline and heated by a lamp. The bile, free from blood, was collected in a test tube and another small test tube was fixed with plaster over the penis to collect urine. After some time, 10 mg. of either nicotinamide or of nicotinamide methochloride were given intraperitoneally.

(*e*) *Effect of faecal bacteria.* An emulsion was made from freshly passed rat faeces, corresponding to about 25 mg. dry weight, in 5 ml. peptone water, and filtered through a sterile paper filter. The filtrate was diluted 100 times with peptone water and 2 drops of this dilution were inoculated into tubes containing a synthetic ammonium lactate medium (Fildes, 1938), or a casein hydrolysate medium (Barton-Wright, 1944), both containing 5 μg./ml. nicotinamide methochloride. Controls were made without nicotinamide methochloride and without bacteria. The tubes were incubated aerobically for 4 days at 37°, the solid was then removed by centrifugation and nicotinamide methochloride was measured in the supernatant fluid. A König (1904) reaction using metol as aromatic amine (Bandier & Hald, 1939) was carried out before and after incubation, and before and after hydrolysis with strong alkali (12 N-KOH), in the presence of ammonia (Perlzweig, Levy & Sarett, 1940).

(*f*) *Effect of hepatic poisons.* Eight rats of both sexes and weighing between 250 and 400 g. were given intraperitoneally 0·2 ml. of a mixture of equal parts of chloroform and carbon tetrachloride twice at 11-day intervals. Four other rats received subcutaneously a dose of 2 ml. of a 0·25% (w/v) solution of yellow phosphorus in peanut oil which was repeated 11 days later. The basal urinary nicotinamide methochloride elimination, the response of these rats to 20 mg. of extra nicotinamide and, in some instances, the urinary total and protein-N output had been measured before the beginning of the poisoning; N estimations were continued during the experiment. The separation of protein and non-protein-N was done by phosphotungstic acid precipitation; N was estimated by a semi-micro-Kjeldahl method.

RESULTS

Most of the results are presented in Tables 1–6 in which all values for nicotinamide methochloride, N and König reactions are averages of two or three separate determinations. There was in both types of experiments reported in Tables 1 and 2 an initial loss, the rate of which is inversely proportional to the concentration. In the liver slice experiments, at any one concentration of added nicotinamide methochloride the loss was greater, the more liver tissue was used. All the loss occurred in the first 2–15 min., no significant further loss being observed during the next 4 hr. This absence of further change after the initial loss cannot be due to an inactivation of the liver tissue by agitation in Krebs-Ringer at 37°, since a corresponding initial loss is seen in exps. 7 and 8, when nicotinamide methochloride was added to the liver-Krebs-Ringer system after a preliminary shaking at 37° for 2 hr.; moreover liver tissue treated in the same manner retains its ability to methylate nicotinamide (Perlzweig, Bernheim & Bernheim, 1943; Ellinger, 1946). The only possible explanation of these results is that an initial adsorption of some nicotinamide methochloride on the liver tissue occurs.

The results on the elimination of nicotinamide methochloride in the faeces (Table 3) are confusing. The daily faecal output varied widely in the same rat, kept under constant dietary and environmental conditions, and the mean figures vary from rat to rat. Considerable doses of either nicotinamide or nicotinamide methochloride, which cause a large rise in the urinary output, did not generally increase the

Table 1. *Effect of sliced liver on nicotinamide methochloride (NM)*

Exp. no.	Wet wt. of liver used (mg.)	Concentration of NM before shaking (μg./ml.)	NM concentration (μg./ml.) after min. shaking: 2	30	60	120	240	Average loss (%)	NM concentration (μg./ml.) of controls after 240 min.: Without NM	Without liver
1	267 (253–278)*	1	0·89	0·90	0·91	0·89	0·90	10·8 } 12·3	0·02	0·99
2	383 (370–397)	1	0·86	0·85	0·88	0·83	0·87	13·8	0·01	1·02
3	282 (269–289)	3	2·82	2·86	2·81	2·86	2·84	5·4 } 7·0	0·3	3·03
4	392 (378–403)	3	2·74	2·72	2·78	2·71	2·76	8·6	0·0	2·96
5	258 (247–266)	10	9·72	9·67	9·77	9·64	9·73	2·9 } 3·25	0·02	10·03
6	314 (299–326)	10	9·65	9·70	9·60	9·58	9·67	3·6	0·01	10·05
7	255 (243–262)	3 NM added after 2 hr. shaking	2·87	2·83	2·84	2·90	—	5·1 } 7·0	0·03	3·05
8	382 (371–395)	3 NM added after 2 hr. shaking	2·72	2·76	2·69	2·74	—	8·9	0·04	3·02

* Values in parentheses are the limiting values.

Table 2. *Nicotinamide methochloride (NM) content of perfusion liquid after passing through a surviving liver preparation*

Exp. no.	Perfusion from	NM content after 20 min. perfusion without NM (μg./ml.)	NM content of perfusion liquid (μg./ml.)	NM content (μg./ml.) of perfusate during min. perfusion: 0–10	10–30	30–60	60–90	Average loss (%)
9	Hepatic artery	0·03	1	0·88	0·90	0·88	0·87	11·8 } 10·1
10		0·02	1	0·92	0·89	0·93	0·92	8·5
11	Portal vein	0·00	1	0·93	0·90	0·91	0·90	9·0 } 10·1
12		0·02	1	0·87	0·89	0·90	0·89	11·2
13	Hepatic artery	0·05	3	2·78	2·80	2·82	2·78	6·8 } 7·0
14		0·02	3	2·83	2·76	2·79	2·80	6·8
15	Portal vein	0·00	3	2·77	2·81	2·77	2·79	7·2 } 7·0
16		0·01	3	2·80	2·76	2·78	2·81	7·1
17	Hepatic artery	0·00	10	9·61	9·58	9·63	9·57	4·0 } 4·1
18		0·03	10	9·57	9·59	9·55	9·56	4·3
19	Portal vein	0·02	10	9·63	9·65	9·60	9·59	3·8 } 4·1
20		0·04	10	9·58	9·55	9·62	9·53	4·3

faecal excretion significantly. The mode of administration of the drugs, oral or intraperitoneal, made no difference to the faecal output, while the urinary elimination was about 50 % higher after intraperitoneal than after oral administration in the case of nicotinamide, and 200–300 % higher in the case of nicotinamide methochloride.

The fluorescent substance developed from the KCl eluate by alkali and *iso*butanol when faeces extracts or bile were tested for nicotinamide methochloride (Tables 3 and 4) gave a greenish fluorescence with strong alkali which faded on standing at room temperature to form a yellowish colour. With dilute potassium ferricyanide the fluorescence became deep violet. Addition of alkali and acetone produced a yellow colour with green fluorescence, thus proving that the fluorescence was due to the presence of 'F_2'.

The examination of bile for nicotinamide methochloride (Table 4) showed clearly that some of this compound formed in the liver was eliminated in the bile, the amount varying between 12·8 and 56 % of that in the urine collected simultaneously. After administration of nicotinamide or nicotinamide methochloride, the output in the bile rose considerably, relatively more in the former than in the latter case, biliary elimination being 92 and 37 %, and 21·7 and 9·3 % of the urinary output, respectively.

Table 3. *Daily faecal elimination of nicotinamide methochloride (NM) before and after oral (O.) or intraperitoneal (Ip.) dosage of 10 mg. nicotinamide (NA) or NM*

Exp. no.	Weight (g.)	Observation period	Limits of daily NM output during pre-dosing period: Urinary (μg.)	Limits of daily NM output during pre-dosing period: Faecal (μg.)	Average NM output on 3 predosing days: Urinary (μg.)	Average NM output on 3 predosing days: Faecal (μg.)	Output 24 hr. after dosing 10 mg. NA: Urinary (mg.)	Output 24 hr. after dosing 10 mg. NA: Faecal (μg.)	Output 24 hr. after dosing 10 mg. NM: Urinary (mg.)	Output 24 hr. after dosing 10 mg. NM: Faecal (μg.)	Route
21	304	19. x.–	78·0–	303·0–	287·2	576·3	1·632	683·0	—	—	O.
		22. xii. 44	366·8	1390·0	313·5	721·4	—	—	1·970	744·0	
					268·3	471·7	2·347	617·2	—	—	Ip.
					303·2	517·3	—	—	5·753	640·2	
22	329	19. x.–	137·3–	278·9–	186·4	346·8	1·287	337·2	—	—	O.
		22. xi. 44	288·7	1017·2	198·6	327·2	—	—	1·246	321·2	
					217·6	412·8	1·964	398·3	—	—	Ip.
					149·6	362·5	—	—	5·483	393·1	
23	362	22. i.–	578·8–	397·2–	672·1	447·6	1·874	430·7	—	—	O.
		14. iii. 45	862·2	768·5	691·7	483·6	—	—	2·168	477·6	
					712·8	507·4	2·583	471·9	—	—	Ip.
					728·6	433·7	—	—	6·821	482·0	
24	369	2. i.–	782·3–	313·6–	848·3	393·6	2·178	425·7	—	—	O.
		27. iii. 45	1104·5	584·7	1009·7	496·2	—	—	2·470	528·3	
					1089·6	557·6	3·176	798·3	—	—	Ip.
					1066·4	568·2	—	—	7·284	673·3	

Experiments nos. 25–28: 4 additional rats treated in exactly the same manner yielded results which did not differ significantly from those of experiments nos. 21–24.

Table 4. *Biliary elimination of nicotinamide methochloride (NM) before and after intraperitoneal dosage with nicotinamide (NA) or NM*

Exp. no.	Weight (g.)	Rate of bile flow during observation period (ml./hr.)	Duration of observation period: Before dosing (min.)	Duration of observation period: After dosing (min.)	Drug dosed	NM output in bile: Before dosing (μg./hr.)	NM output in bile: After dosing (μg./hr.)	Simultaneous urinary output of NM: Before dosing (μg./hr.)	Simultaneous urinary output of NM: After dosing (μg./hr.
29	257	0·86	30	210	NA	11·3	183·7	32·3	198·3
30	313	0·61	30	180	10 mg.	3·8	86·2	29·7	232·0
31	287	0·83	30	240		6·7	98·0	22·4	450·7
32	186	0·69	30	180	NM	18·0	35·1	14·3	378·6
33	153	0·71	30	150	10 mg.	8·9	83·2	—	—
34	211	0·77	15	90		7·2	77·3	—	—

Table 5. *Effect of incubation with faecal bacteria on nicotinamide methochloride (NM) solutions*

Exp. no.	Dry wt. of faeces (mg.)	NM content of ammonium lactate medium: Before incubation: C.E.H.* (μg./ml.)	Before incubation: König† (μg./ml.)	After incubation: C.E.H.* (μg./ml.)	After incubation: König† (μg./ml.)	Medium alone after incubation (μg./ml.)	NM content in Barton-Wright medium: Before incubation: C.E.H.* (μg./ml.)	Before incubation: König† (μg./ml.)	After incubation: C.E.H.* (μg./ml.)	After incubation: König† (μg./ml.)	Medium alone after incubation (μg./ml.)
35	24·7	5·13	5·32	1·09	1·23	4·89	—	—	—	—	—
36	27·8	5·22	5·27	0·39	0·45	5·11	—	—	—	—	—
37	29·6	5·13	5·44	0·53	0·55	5·21	—	—	—	—	—
38	24·1	—	—	—	—	—	5·18	5·52	0·84	0·90	5·32
39	30·6	—	—	—	—	—	4·98	5·63	0·53	0·58	4·97
40	28·7	—	—	—	—	—	4·86	5·49	0·29	0·35	4·91

All König (1904) reactions before hydrolysis gave no measurable colour.

* Determined by the method of Coulson, Ellinger & Holden (1944).

† Determined by the König method after hydrolysis with strong alkali in presence of ammonia.

Table 6*a*. *Effect of liver poisons on the basal urinary elimination of nicotinamide methochloride (NM) and on the response to ingested nicotinamide (NA)*

Exp. no.	Weight (g.)	Poison used	Urinary pre-poisoning NM output: mean of 3 days (μg./day)	Response before poisoning to 20 mg. NA (mg./day)	First poisoning period Urinary NM output (μg./day) after poisoning on day 1	2	3	4	5	6*	7	8–10 aver.
41	287	$CHCl_3$ (0·1 ml.) + CCl_4 (0·1 ml.)	168·7	5·037	517·0	179·0	134·3	121·2	138·7	2264·0	490·7	155·6
42	318		328·6	4·807	867·0	432·0	310·1	151·7	183·9	1976·2	702·0	322·1
43	396		276·0	4·708	1068·0	632·1	260·3	210·7	201·6	1876·3	658·3	316·0
44	274		874·3	5·237	1924·0	1160·1	840·1	621·5	578·2	2174·0	917·3	790·6
45	343	P (2 ml. of 0·25 % soln.)	763·5	5·198	1632·0	1072·3	694·3	584·2	579·0	1870·0	937·4	780·7
46	317		212·8	4·317	1216·2	485·7	208·3	154·7	137·8	1674·2	516·2	198·6

Exp. no.	Weight (g.)	Poison used	Urinary pre-poisoning NM output: mean of 3 days (μg./day)	Response before poisoning to 20 mg. NA (mg./day)	Second poisoning period Urinary NM output (μg./day) after poisoning on day 1	2	3	4	5	6*	7
41	287	$CHCl_3$ (0·1 ml.) + CCl_4 (0·1 ml.)	—	—	552·0	216·0	140·7	110·3	121·0	2603·0	461·2
42	318		—	—	759·0	388·6	217·1	209·3	224·3	2016·0	754·6
43	396		—	—	923·3	472·4	240·3	198·7	196·8	1948·0	834·0
44	274		—	—	1763·0	1064·0	742·0	533·1	542·8	1843·2	767·0
45	343	P (2 ml. of 0·25 % soln.)	—	—	1974·0	Dead	—	—	—	—	—
46	317		—	—	1374·0	873·2	Dead	—	—	—	—

Nos. 47–52: 4 additional rats were poisoned with the chloroform-carbon tetrachloride mixture and 2 with yellow phosphorus in exactly the same way. N elimination was not measured. The NM elimination and the response to NA was not significantly different from those reported above.

* After intraperitoneal injection of 20 mg. NA.

Table 6*b*. *Effect of liver poisons on the urinary elimination of total and protein nitrogen (TN and PN)*

Exp. no.	Poison used	Urinary N before poisoning mean of 3 days TN (mg./day)	PN (mg./day)	First poisoning period Urinary N output (mg./day) after poisoning on day 1 TN	PN	2 TN	PN	3 TN	PN	4 TN	PN	5 TN	PN	6 TN	PN	7 TN	PN	8–10 aver. TN	PN
41	$CHCl_3$ (0·1 ml.) + CCl_4 (0·1 ml.)	81·2	4·9	92·3	14·8	87·2	6·8	82·0	5·0	—	—	—	—	—	—	82·1	4·7	83·2	4·8
42		90·5	5·8	107·2	17·2	95·6	10·4	91·6	6·1	—	—	—	—	—	—	91·0	6·1	90·7	6·3
43		132·2	7·3	148·7	22·6	136·3	10·9	133·1	9·0	—	—	—	—	—	—	133·7	7·5	131·5	7·2
44		76·3	4·4	89·3	12·4	81·2	6·2	77·0	4·1	—	—	—	—	—	—	75·8	4·2	74·0	4·3
45	P (2 ml. of 0·25 % soln.)	117·8	6·5	129·8	17·9	123·2	11·5	118·0	6·8	—	—	—	—	—	—	118·3	6·9	121·2	7·2
46		92·5	6·3	107·3	18·4	96·8	10·3	91·9	6·5	—	—	—	—	—	—	92·3	6·5	93·1	6·3

Exp. no.	Poison used	Second poisoning period Urinary N output (mg./day) after poisoning on day 1 TN	PN	2 TN	PN	3 TN	PN	4 TN	PN	5 TN	PN	6 TN	PN	7 TN	PN
41	$CHCl_3$ (0·1 ml.) + CCl_4 (0·1 ml.)	94·7	17·3	89·3	8·3	81·4	5·2	—	—	—	—	—	—	82·0	4·8
42		104·3	14·8	96·8	8·7	91·4	5·8	—	—	—	—	—	—	91·8	6·1
43		146·3	18·7	135·2	9·7	133·6	7·2	—	—	—	—	—	—	134·2	7·5
44		186·2	10·8	79·6	7·2	77·0	4·3	—	—	—	—	—	—	75·8	4·4
45	P (2 ml. of 0·25 % soln.)	131·3	19·4	Dead	—	—	—	—	—	—	—	—	—	—	—
46		109·4	19·2	99·3	14·6	Dead	—	—	—	—	—	—	—	—	—

To obtain the six results quoted in Table 4, 15 animals had to be used. In some cases extra nembutal had to be given to keep the animal quiet and this sometimes caused death from respiratory paralysis. Moreover, in some animals it was almost impossible to find the common bile duct which was hidden in the mesenteric fat. An uninterrupted flow of bile could only be obtained if the intestines were handled very gently. Urine was passed spontaneously only by one animal; in three more it was possible to obtain urine by pressure on the bladder. It is noteworthy that the relative biliary output is much higher when nicotinamide methochloride is formed in the liver from nicotinamide than when the methylated compound is ingested.

Table 5 shows that an emulsion of intestinal bacteria growing aerobically in various media destroyed between 80 and 94% of added nicotinamide methochloride within 4 days.

In exps. 41–44 (Tables 6*a*, 6*b*) rats were killed on the 8th day after the last dose of chloroform-carbon tetrachloride mixture. No pathological lesions could be observed in the livers. In exps. 45 and 46, the livers were examined immediately after death and found on macroscopical examination to be diffusely yellow and swollen; microscopical examination (magnification 16 ×) indicated that the acini were indistinct.

The experiments on the effect of liver poisons (Table 6*a*, 6*b*) showed, instead of the expected diminished output after poisoning, a transitory rise in the urinary nicotinamide methochloride elimination and a simultaneous rise in that of protein-N. The former then fell below the predosing level, the latter to the predosing level; 8–10 days after the poisoning, both were back to normal. The same occurred when the rats were poisoned for a second time with chloroform-carbon tetrachloride 11 days after the first dose, the animals recovering. But a second poisoning with phosphorus caused death with severe hepatic degeneration. If on the 5th day after poisoning, 20 mg. of nicotinamide were injected, the response in all cases was less than half of that observed before poisoning, indicating a severe damage to the methylating mechanism.

DISCUSSION

The fate of nicotinamide methochloride in the mammalian body is still obscure. There is no direct evidence for the statement by Perlzweig, Huff & Rosen (1946) that nicotinamide methochloride is destroyed in the liver. In man, parenterally administered nicotinamide methochloride is eliminated in the urine to about 60% and the remainder cannot be traced; in the rat, the urinary elimination of orally ingested nicotinamide methochloride is temporarily increased by about 50% after poisoning of the liver with carbon tetrachloride. These findings led Perlzweig, Huff & Rosen (1946) to conclude that poisoning of the liver diminished a normal hepatic destruction of nicotinamide methochloride. Najjar & Deal's (1946) suggestion of a demethylation of nicotinamide methochloride in the liver is based on even more indirect evidence. From Tables 1 and 2 it is clear that surviving liver tissue or perfused surviving liver do not destroy significant amounts of nicotinamide methochloride. Destruction of nicotinamide by liver *in vivo* would thus appear improbable.

It is difficult to relate the results reported in Table 4 of liver and kidney function in the rat on a time basis. The rat has no gall bladder and no bile is stored; some urine, however, may be stored for many hours in the bladder and a complete collection is difficult. It is, therefore, impossible to obtain over a short period a complete balance sheet of the relative elimination of nicotinamide methochloride in bile and urine; collection of bile over a 24 hr. period seems hardly possible in the rat.

The experiment reported in Table 5 on bacterial destruction of nicotinamide methochloride *in vitro* suggests that a similar destruction may occur in the intestinal tract during life. The great difference in urinary elimination of nicotinamide methochloride when the drug is given by mouth as compared with the values obtained on injection, which is shown here for the rat (Table 3) and has been demonstrated for man in earlier papers (Ellinger & Coulson, 1944; Perlzweig & Huff, 1945), is probably due to this destruction in the intestines. The negative König reaction in the medium before hydrolysis shows that even the pyridine ring is disrupted by the bacteria. There is in addition some formation of nicotinamide methochloride from nicotinamide by *Proteus vulgaris* (Ellinger & Abdel Kader, 1947). This extensive destruction and small scale formation of nicotinamide methochloride by the intestinal bacteria explain the results of the examination of the faecal output. Whether such destruction accounts for the balance of nicotinamide methochloride, eliminated neither in urine nor in faeces, cannot be decided from these experiments, but it is probable that most or all of the nicotinamide methochloride not accounted for by the urinary output is eliminated in the bile and then for the most part destroyed by the bacteria of the lower intestines; some may be reabsorbed and the remainder eliminated in the faeces. A complete balance of the nicotinamide methochloride metabolism requires information about the factors which determine the relative elimination in bile and urine and as to whether this distribution between bile and urine is constant or varies between different individuals and changes in the same animal under various conditions. Such knowledge cannot be gained from the experiments reported;

this could only be done if arrangements can be made to collect bile and urine simultaneously over a long period. The increased spontaneous nicotinamide methochloride output reported in Table 6*a* during the first day following administration of poison is probably due to an increase of available nicotinamide caused by tissue destruction and the liberation of coenzymes. Such tissue disintegration is proved by the increased output of protein nitrogen. No indication of a decreased destruction of nicotinamide methochloride can be found and the temporary increase in urinary elimination of ingested nicotinamide methochloride after carbon tetrachloride poisoning found by Perlzweig, Huff & Rosen (1946), can be fully explained by the increase of the circulating nicotinamide from tissue breakdown and the diminished elimination of the methylated compound in the bile from the damaged liver.

SUMMARY

1. Experiments carried out *in vitro* produced no evidence that destruction of nicotinamide methochloride occurred in the liver.

2. It could be shown that a considerable portion of nicotinamide methochloride formed in the liver or administered to a rat, was eliminated with the bile into the intestinal tract. Whether this accounts for the total amount, not eliminated in the urine, could not be decided.

3. An emulsion of intestinal bacteria destroyed a large part of the nicotinamide methochloride present in the medium.

4. The faecal elimination of nicotinamide methochloride depends on the elimination in the bile, its destruction and synthesis by intestinal bacteria and perhaps on reabsorption from the intestines. Faecal output affords no conclusive evidence regarding the nicotinamide state of the body.

5. Liver poisons such as chloroform, carbon tetrachloride or yellow phosphorus cause a temporary rise in nicotinamide methochloride output, probably due to tissue destruction, but they also reduce temporarily the efficiency of the methylating mechanism of the liver.

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The Cystine and Methionine Contents of Liver Protein in Acute Hepatic Necrosis

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Acute necrosis of the liver has been produced in rats by many workers using diets deficient in the sulphur-containing amino-acids. This work has recently been reviewed by Glynn, Himsworth & Neuberger (1945). All have found that there is a delay of 20–100 days, during which the animal appears normal, before the sudden onset of the disease. The condition has been attributed to a simple deficiency of cystine, although methionine can replace cystine in preventing the disease. It has been suggested that during the latent period the animal is living on endogenous stores of cystine and methionine and that the onset of disease follows their final depletion. It is possible that an unknown

positive factor is also involved. An infection seems to have been excluded by Weichselbaum (1935) and by Himsworth & Glynn (1944*a*). Hock & Fink (1943) favour on histological grounds an explanation involving the action on the liver of a poisonous substance from the gut which requires cystine for its detoxication. This is supported by the work of Weichselbaum (1935) who was able quickly to revive rats dying from acute hepatic necrosis by feeding 100 mg. of cystine by stomach tube, or by an intraperitoneal injection of 150 mg. of glutathione. It is difficult to imagine the cystine being used to form protein under these conditions, as relatively large amounts of other amino-acids, including methionine, would also be simultaneously required, and could not be immediately available.

Clearly the changes in sulphur content of the liver that may be taking place during the latent period of the disease and after it has become established may provide information as to its mechanism. The question of the possible 'plasticity' of the liver proteins arises. This subject has been reviewed by Luck (1937) who considers that the matter is not yet proved either way. Several protein fractions have been isolated from the liver and a change in their relative proportions as well as in their individual composition could cause gross changes in the content of sulphur-containing amino-acids. No work on this subject has yet been reported with regard to acute hepatic necrosis produced by dietary deficiencies. Some attention has, however, been paid to the problems arising from directly administered liver poisons.

The histological appearance in the latter case is usually that of a zonal necrosis of liver cells, i.e. it is limited to particular zones of each lobule throughout the liver, as in the centrilobular necrosis following chloroform poisoning. In dietary necrosis on the other hand the necrosis tends to be massive in type, i.e. it affects entire lobules in certain parts while leaving them comparatively unharmed elsewhere. However, in spite of these histological differences the two problems resemble one another strongly in that both diseases are favoured by dietary deficiencies in the sulphur-containing amino-acids. Hence, there is good reason to suppose that there is in both cases a similar protective mechanism involving sulphur.

Miller & Whipple (1942) claimed that they could raise the gross nitrogen/sulphur (N/S) ratio of dog's liver from 11–12 : 1 to 14–15 : 1 by the giving of a low protein diet. In this condition of relatively low sulphur content the livers were easily damaged by chloroform. Giving methionine lowered the ratio to normal, or even below normal (i.e. increased the relative amounts of sulphur) and increased the resistance to chloroform. Several amino-acids not containing sulphur were tried without any similar effect. Cystine had a weaker protective action. The high resistance of the liver of the newborn pup towards chloroform was ascribed to its unusually low N/S ratio. Miller & Whipple (1942), however, determined only the total sulphur and nitrogen in the dried livers, so they could not have detected any change in the relative contributions of protein or non-protein sulphur to these figures, nor any change in the relative cystine or methionine contents.

More recently Shaffer & Critchfield (1945) have analyzed the livers of rats on a sulphur-poor diet after several days of carbon tetrachloride inhalation. They found no change in the N/S ratio of dried fat-free liver.

It seemed desirable therefore to study the possible changes in the cystine and methionine contents of pure total liver protein in dietary acute hepatic necrosis. Suitable material from rats on a synthetic diet in which mixtures of pure amino-acids were used instead of protein was available from experiments being carried out by Glynn, Himsworth and Neuberger (1945). Analyses were also carried out on the liver protein obtained from a strain of black-hooded rats which had appeared to be more resistant than other strains to the disease, for comparison with the corresponding figures for susceptible white Wistar rats.

EXPERIMENTAL

Analytical methods. Cystine and methionine were determined by the gravimetric method of Lugg (1938). This requires three separate operations followed in each case by precipitation and weighing of the liberated sulphur as $BaSO_4$. Total sulphur (*A*) is determined after fusion of the protein with sodium peroxide and carbonate, cystine plus sulphate sulphur (*B*) after refluxing with fuming nitric acid, and sulphate sulphur (*C*) after complete acid hydrolysis of the protein. Clearly *A*–*B* = methionine sulphur, while *B*–*C* = cystine sulphur. The last determination (*C*) was omitted in the second experiment. Lugg's method was tedious, but gave good results with pure methionine and cystine, and duplicates on proteins agreed well. Its accuracy is subject to the assumption that there is no source of sulphur in the protein apart from methionine, cystine and free sulphate. Nitrogen was determined throughout by the micro-Kjeldahl method.

The baker's yeast used in the first experiment had on analysis the following composition: total sulphur 0·53, 0·49, 0·52%; cystine plus sulphate sulphur 0·19, 0·19%; sulphate sulphur 0·03, 0·03%; whence methionine = 1·49%, cystine = 0·60%. Total N = 7·02%. Total N/Total S = 13·8 : 1. The yeast contained a higher proportion of its sulphur as methionine than was expected (Himsworth & Glynn, 1944*b*, footnote p. 137). Block & Bolling (1945), however, recently obtained only slightly lower figures than these for both amino-acids. In the usual average daily intake of 8 g. total food, containing 1·3 g. of crude yeast, each rat received 19·4 mg. of methionine and 7·8 mg. of cystine.

Animals and diets. In the first experiment livers from white Wistar and black-hooded male rats, were used. These animals had been fed in groups of eight on a diet consisting essentially of the low fat, 8% yeast protein diet described

by Himsworth & Glynn (1944*a*). They averaged 100 g. in weight at the beginning of the experiment.

In the second experiment, material from white Wistar male rats was used. These averaged 80 g. in weight at the beginning of the experiment. Except for the control group on casein, the dietary nitrogen was entirely supplied by mixtures of pure amino-acids (see Table 1). Full details of the diets are reported elsewhere (Glynn *et al.* 1945). The rats were separately fed and the protein, or amino-acid, intake was kept constant by reducing only the carbohydrate intake if the appetite was poor. Adequate vitamin and mineral supplements were given in all experiments.

Table 1. *Protein and amino-acid composition of diets in second experiment*

Group 1	3 rats (I/1–I/3), casein 0·86 g.
Group 2	4 rats (II/1–II/4), sulphur-free amino-acid mixture 1·3 g. + methionine 80 mg.
Group 3	3 rats (III/1–III/3), sulphur-free amino-acid mixture 1·3 g. + cystine 64 mg.
Group 4	3 rats (IV/1–IV/3), sulphur-free amino-acid mixture 1·3 g.
Group 5	4 rats (V/1–V/4), sulphur-free amino-acid mixture 1·3 g. + methionine 8 mg.

The 1·3 g. of amino-acid mixture contained pure amino-acids in the same quantities as in the 0·86 g. of casein except for methionine and cystine. The individual rats are designated as shown in brackets.

Two animals from each of the Groups 1 and 2 (namely I/2, I/3, II/3 and II/4) were killed at about 100 days. The remaining animals from these groups (namely I/1, II/1 and II/2) were then given the basal sulphur-free diet (Group 4, Table 1).

Technique of sampling of livers. In the first experiment, several rats happened to die on the same day with acute necrosis of the liver. They had been 118 days on the diet. This enabled the bulking of the livers so as to provide ample amounts of protein for analysis. It also made possible the perfusion with saline before working up as usual of another group of necrotic livers from animals in the same experiment. This was considered important. Even in normal livers the blood unavoidably left behind makes a variable contribution to the protein content. In acute necrosis of the liver there is good evidence (Himsworth & Glynn, 1945) of additional infiltration of the liver with fluid, probably plasma, containing protein. After removal of necrotic livers considerable oozing of serum from the inferior vena cava was commonly seen. Losses in weight of as much as 1·5 g. in a liver weighing 5·8 g. when first removed were caused in this way. The oozing appeared to be greatest when the onset of the disease was most acute. Clearly if this extra protein differed markedly from liver protein in its sulphur-containing amino-acid content it would be impossible to distinguish analytical differences due to the liver necrosis from those due to this variable amount of adventitious protein. In two of the liver perfusions the amount of haemoglobin washed out was roughly estimated in the Haldane apparatus and the total nitrogen determined. In the first liver (wt. 11·7 g.) the haemoglobin corresponded to the amount contained in 0·75 ml. of normal blood (Hb 100%) while the amount of nitrogen washed out was 15% of the total nitrogen remaining in the liver. In the second liver (wt. 7·3 g.) the figures were 0·5 ml. and 20% respectively. Some loss of actual liver protein would also be expected under the conditions of operation.

On the following day livers were obtained from some apparently normal rats surviving on the same diet, which were killed as controls. Some black-hooded rats, also normal, were killed and their livers analyzed. The results are given in Tables 2 and 3.

These experiments showed that perfusion of the liver lowered the non-protein nitrogen but did not affect the cystine or methionine content of the protein obtained (Table 2). No further perfusions were therefore considered necessary.

Isolation of liver protein. The rats were killed by stunning, quickly opened and bled into the pleural cavities by cutting the inferior vena cava. When the heart had stopped beating the liver was quickly removed, weighed and dropped into 50 ml. of boiling water. After 15 min. gentle boiling it was removed and ground with 20 times its weight of 10% (w/v) trichloroacetic acid. It was then centrifuged in a 250 ml. bottle, the supernatant liquid poured off, and the residue washed successively with hot water, hot ethanol containing 10% (v/v) ether and light petroleum (b.p. 40–60°). The aqueous washings were combined with the water used for boiling the liver and analyzed for non-protein nitrogen by the Kjeldahl method. The ethanol and ether washings were evaporated to dryness and the residue extracted with light petroleum. The residue from evaporation of this extract was weighed as fat. The insoluble liver protein, usually a fine buff powder, was dried at 100° to remove solvents, finely ground and then left exposed for several days till it had attained constant weight. It was then ready for analysis. In the three rats IV/1, IV/2 and V/4 (marked † in Table 4) the livers were not worked up in this way. They were rapidly dried *in vacuo* at 100°, then finely ground and thoroughly extracted with ether. The dried residue was analyzed as such. It probably contained a little remaining non-protein material.

The livers of rats I/2 and I/3 were combined for analysis, the growth curves of the rats having agreed very closely. Similarly the livers of II/3 and II/4 were combined. The sulphate sulphur was not determined for any of these rats, owing to the small amount of protein available. It was assumed throughout to equal 0·04%.

Liver perfusions. These were carried out immediately after death. The procedure was as above except that before removal of the liver from the abdomen, a small needle was tied into the portal vein. The liver was then removed with the portal vein intact and the needle *in situ*. Isotonic saline was allowed to pass at room temperature and at a few cm. pressure for about 10 min. by which time the effluent from the cut ends of the vena cava was clear and colourless and the liver had become noticeably paler although the red patchy subcapsular 'haemorrhages' were not washed out. The liver bulged evenly during the perfusion and the lobular pattern was evenly exaggerated throughout, suggesting adequate perfusion of the saline in the vascular pathways.

Water content of tissues. In all the rats in the second experiment a slice of the liver was taken for drying at 105° to constant weight and the residue analyzed for total nitrogen by the Kjeldahl method. Loss of weight at 105° of a slice of kidney and of adductor muscle was also determined as a matter of routine, as a check on generalized

oedema. These routine liver analyses confirmed the gross chemical changes found by Himsworth & Glynn (1945). The necrotic livers were heavier than normal, the ratio, liver weight/body weight × 100 being constantly greater than 5. It was less than 4 in the non-necrotic livers. An increase in g. water/100 g. liver was also found in the necrotic livers and also a rather slighter increase in the total liver protein (N × 6·25)/100 g. body weight. The fat content of the livers varied from 0·6–7·0% and bore no relation to the presence of necrosis.

RESULTS

The results are given in Tables 2–5.

However, it is believed that some definite conclusions can be drawn from the relatively few experiments described here. In the first experiment with the yeast diet, the detailed analyses (Table 3) show no significant difference in cystine and methionine contents between the normal black-hooded and the Wistar rats such as might have accounted for the different resistance of their livers to the disease. The cystine contents of the liver proteins were very constant and were independent of the presence or absence of necrosis of the liver. The methionine content, however, was a little higher in the normal animals than in those with necrosis. The gross differences in N/S ratio were much smaller than those found by Miller & Whipple (1942) in their dogs. As the rats were group-fed there may have been wide variations in individual food intake especially during the day before the normal animals were killed, as there was available to them the food left over by the rats dying from acute necrosis. It is not believed, therefore, that any further conclusions can be formed from this experiment.

Table 2. *Gross liver analyses in first experiment with yeast diet*

Group	No. of rats grouped	Body wt. when killed (g.)	State of livers	Whether perfused	Fat (g./100 g. fresh liver)	NPN (g./100 g. fresh liver)	Protein obtained from livers (g.)	N (g./100 g. liver protein)
A. White Wistar rats	3	130, 108, 84	Acute necrosis	No	5·7	0·12	3·65	14·1
B. White Wistar rats	4	112, 100, 161, 91	Acute necrosis	Yes	7·1	0·08	3·92	14·3
C. White Wistar rats	2	165, 174	Normal	No	5·2	0·17	2·15	13·8
D. Black-hooded rats	2	124, 133	Normal	No	5·4	0·13	1·85	13·6

Table 3. *Methionine and cystine content in g./100 g. of protein isolated from livers in the first experiment with the yeast diet*

Group	Total S	Cystine + SO_4-S	SO_4-S	∴ Methionine	∴ Cystine	Total N/ Total S
A. White Wistar rats	1·33	0·43	0·04	4·16	1·48	12·0
B. White Wistar rats	1·33	0·43	0·04	4·18	1·44	12·0
C. White Wistar rats	1·46	0·43	0·06	4·77	1·38	11·0
D. Black-hooded rats	1·47	0·44	0·05	4·76	1·45	10·9

The figures are all calculated on the basis of g./100 g. of protein containing 16% N.

DISCUSSION

The object of this investigation was to secure evidence in favour of the theory that acute hepatic necrosis is a simple deficiency disease due to lack of the two sulphur-containing amino-acids in the diet. Strong evidence would be obtained if it could be shown that the latent period in the development of the disease and the final diseased condition of the liver were closely related to decreases in the total quantity of one or other amino-acid. An 'ideal' experiment could be devised in which rats would be separately fed on synthetic diets with pure amino-acids in place of protein. Some would then be killed in groups during the latent period of the disease. These livers, as well as those obtained after death from acute necrosis, would be analyzed. Even under these conditions, however, the rats would be expected to die of acute necrosis of the liver at widely varying times owing to the existence of unknown factors in the pathogenesis of the disease (see Table 4; larger variations and even complete failure to produce the disease have also been found with yeast and casein diets). Large numbers of animals would therefore be required to average out these variations. The cost of the experiment would be prohibitive.

The results of the second experiment with the synthetic diet show only slight variations in the cystine content of the liver protein and more marked changes in methionine (Table 4). At first sight the figure of 7·15% methionine in the necrotic liver of rat IV/3 contrasts markedly with those of 4·64 and 5·07% for the healthy controls I/2+I/3 and II/3+II/4 which were fed on a diet adequate for growth. On the other hand, similarly high methionine values were found in the liver protein from rats III/2 and III/3 which did not get necrosis but appeared to die of anaemia and hypoproteinaemia. It seems that the chief factor here is not the presence or absence of acute necrosis but the state of development of the animal. The high methionine values occurred in the small livers of the rats that had failed to grow

Table 4. *Methionine and cystine content in g./100 g. of protein isolated from livers of rats in the second experiment with the amino-acid diet*

Rat	Diet	Diet fed days	Weight of rat when killed (g.)	State of livers	Protein isolated from liver (g.)	N (g./100 g. protein)	Total S*	Cystine and SO_4-S*	∴ Methionine*	∴ Cystine*	Total N/ Total S
I/1	Casein then sulphur-free	130 and 18 respectively	118	Necrosis	0·61	13·9	1·74	0·58	5·37	1·73	9·2
I/2 and I/3	Casein	108	181 and 200	Normal	2·30	14·4	1·60	0·51	5·07	1·55	10·0
II/1	High methionine then sulphur-free	118 and 22 respectively	119	Necrosis	0·59	13·8	1·76	0·57	5·48	1·70	9·1
II/2	High methionine then sulphur-free	118 and 8 respectively	178	Necrosis	1·24	13·7	1·73	0·60	5·25	1·77	9·2
II/3 and II/4	High methionine	101	222 and 225	Normal	2·75	14·7	1·51	0·52	4·64	1·61	10·6
III/1	High cystine	146	43	Normal	0·42	14·0	1·72	0·55	5·43	1·65	9·3
III/2	High cystine	98	41	Normal	0·33	14·5	2·24	0·64	7·45	2·01	7·1
III/3	High cystine	57	61	Normal	0·44	13·8	2·19	0·61	7·33	1·82	7·3
IV/1†	Sulphur-free	33	47	Necrosis	0·26	13·1	1·72	—	—	—	9·3
IV/2†	Sulphur-free	21	57	Normal	0·44	13·5	1·68	0·62	4·97	1·78	9·5
IV/3	Sulphur-free	57	55	Necrosis	0·30	13·0	2·16	0·62	7·15	1·72	7·4
V/1	Low methionine	44	74	Necrosis	0·44	12·5	2·07	—	—	—	7·7
V/2	Low methionine	103	92	Post-necrotic scarring	0·72	14·5	—	0·56	—	1·74	—
V/3	Low methionine	38	94	Necrosis	0·44	15·2	1·70	0·53	5·42	1·74	9·4
V/4†	Low methionine	34	95	Necrosis	0·88	12·3	1·79	—	—	—	8·9

* The figures for sulphur and amino-acids are all calculated on the basis of g./100 g. of protein containing 16% of N.
† Liver protein obtained by drying at 100° followed by ether extraction.

Table 5. *Sulphur, methionine and cystine content in g./100 g. of protein isolated from livers of rats in the second experiment with the amino-acid diet*

Rats rearranged in order of length of time on methionine-free or low methionine diets

Rat no.	I/2+I/3	II/3+II/4	II/2	I/1	II/1	IV/1	V/4	V/3	V/1	IV/3	III/3	III/2	III/1
Body weight at death (g.)	181 and 200 respectively	222 and 225 respectively	178	118	119	47	95	94	74	55	61	41	43
Days on methionine-free or low methionine diet	0	0	8	18	22	33	34	38	44	57	57	98	146
Sulphur (g./100 g.)*	1·60	1·51	1·73	1·74	1·76	1·72	1·79	1·70	2·07	2·16	2·19	2·24	1·72
Methionine (g./100 g.)*	5·07	4·64	5·25	5·37	5·48	—	—	5·42	—	7·15	7·33	7·45	5·43
Cystine (g./100 g.)*	1·55	1·61	1·77	1·73	1·70	—	—	1·74	—	1·72	1·82	2·01	1·65
N/S ratio of liver protein	10·0	10·6	9·2	9·2	9·1	9·3	8·9	9·4	7·7	7·4	7·3	7·1	9·3
Cause of death	Healthy controls, killed		Acute necrosis of liver								Anaemia and hypoproteinaemia		

* The sulphur and amino-acid contents are calculated on the basis of g./100 g. of protein containing 16% N.

because they had been on a diet lacking in the essential amino-acid methionine. This deduction is borne out also in the rats of Groups 1 and 2 (i.e. rats I/1, II/1 and II/2) which were put on the basal sulphur-free diet after they had grown normally on the adequate casein and high methionine diets respectively. Rat II/2 died of typical acute hepatic necrosis in the record time of only 8 days on the deficient diet. Its weight had only fallen from 206 to 178 g. during that time. By comparing the liver analysis for this rat with those for the control animals II/3+II/4 we can determine the effect of the disease itself with less interference than usual by other variables such as body weight and previous dietary history. The analyses are not significantly different. In Table 5 the analyses are re-arranged in order of the length of time during which the rat had been on the methionine-free (Groups 3 and 4) or low methionine (Group 5) diets. The analyses are now roughly in order of increasing sulphur and methionine contents and of decreasing N/S ratio. There is a less marked tendency for the cystine content to rise also. The only notable exception is the remarkable rat III/1 which survived 146 days on a diet rich in cystine but completely devoid of methionine and which only lost 7 g. in weight in the last 50 days. It appears to have made no difference to the composition of the liver protein if the daily diet contained no sulphur (Group 4), 8 mg. of methionine (Group 5) or 64 mg. of cystine (Group 3). Moreover the general trend of analytical results is quite independent of whether or not the rat died of acute hepatic necrosis. The conclusion is drawn that there is no change in cystine or methionine content of the liver protein characteristic of the dietetic necrosis as such.

The possibility that there may be gross changes in the content of other amino-acids has been briefly tested. As a rough check the two liver proteins from rats II/3+II/4 (normals) and from rat II/2 (acute hepatic necrosis) were hydrolyzed for 24 hr. with approx. 5N-hydrochloric acid under identical conditions. Equal quantities of the hydrolyzates were then run on the two-dimensional paper chromatograms of Consden, Gordon & Martin (1944) using the pairs of solvents phenol/collidine and butanol-benzyl alcohol/collidine. The former gives a better separation of the low molecular weight amino-acids and the latter distinguished best between the larger molecules. The developed chromatograms were almost identical as to the size, strength and position of all the amino-acids shown.

The results obtained here can be reconciled with those of Miller & Whipple (1942) by assuming that the changes in total sulphur and N/S ratio they recorded are entirely referable to changes in the non-protein part of the liver. That such changes can occur has been shown by Lee & Lewis (1934) who analyzed separately for total sulphur the non-protein and protein fractions of the liver of rats who had received cystine. They found sharp rises in non-protein sulphur, but the liver protein was unchanged. The work of Brunschwig, Nichols, Bigelow & Miles (1945) also supports the existence of a similar mechanism. These workers found that in dogs liver damage due to chloroform, as measured by impaired bromsulphthalein excretion, could be lessened by injections of sodium thioglycollate as well as of methionine. They suggested that the protective action was due to the donation of —SH groups. However the thioglycollate may act, the reaction must occur in the non-protein fraction as, unlike methionine, it cannot be built up into body protein. The comparison between dogs and rats, however, is subject to criticism on the grounds of the possibility of species differences. The recent work of Leaf & Neuberger (1947), who find a marked fall in the glutathione and total —SH in the non-protein nitrogen of the liver of rats after about 10 days on a diet low in sulphur-containing amino-acids, points more directly to the same conclusion.

SUMMARY

1. Analyses for cystine and methionine of the liver protein from rats with acute hepatic necrosis are reported. The diets covered a wide range of intake of cystine and methionine.

2. It is concluded that the development of acute necrosis is not related to a change in the proportion or amount of these amino-acids in the liver protein.

3. The methionine content of the liver protein from rats maintained on diets devoid of methionine, until they lost much weight was increased, but the cystine content rose only slightly.

4. These results can be best reconciled with previous work on liver damage caused by chlorinated hydrocarbons, by assuming that the significant changes, if any, in total sulphur of the liver are referable only to the non-protein fraction.

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* The sulphur and amino-acid contents are calculated on the basis of g./100 g. of protein containing 16% N.

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